INTERSPECIFIC CROSS-INCOMPATIBILITY BETWEEN *Vaccinium corymbosum* L. AND *V. elliottii* CHAPM: CAUSES AND ATTEMPTS TO OVERCOME THE HYBRIDIZATION BARRIERS

By

CARLOS E. MUÑOZ

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
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INTERSPECIFIC CROSS-INCOMPATIBILITY BETWEEN *Vaccinium corymbosum* L. AND *V. elliottii* CHAPM: CAUSES AND ATTEMPTS TO OVERCOME THE HYBRIDIZATION BARRIERS

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Carlos E. Muñoz

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Chairman: Paul M. Lyrene
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Vaccinium elliottii is a diploid blueberry species with potential for improving cultivars adapted to north-central Florida. Hybrids between *V. elliottii* and the cultivated tetraploid blueberry, *V. corymbosum*, are extremely difficult to obtain. Since a relatively large number of hybrids are needed in a breeding program, research was conducted to study the factors that reduce the number of hybrids obtained and to evaluate several *in vitro* techniques that might enhance hybrid production.

Pre- and postfertilization barriers were detected. Prefertilization barriers were only observed when *V. elliottii* was the male parent and manifested as an arrest of pollen tube growth at the

base of the style. When fertilization occurred normally, in either *V. corymbosum* X *V. elliottii* or its reciprocal cross, a strong postfertilization barrier was detected during early seed development. The zygote remained in a resting stage following fertilization and usually aborted before dividing. The endosperm went into 4-6 cycles of cell division before it started to disintegrate. Endosperm malfunction and embryo abortion were delayed when *V. corymbosum* was the female parent.

Attempts to overcome the incompatibility using *in ovule* and *in ovary* embryo rescue resulted in the production of few presumable hybrid seed, but at a rate no greater than when normal crossing procedures are used. Addition of various vitamins, amino acids, and growth regulators did not increase seed production. Abortion was delayed and the zygote remained alive but undivided in cultured embryo sacs.

Seed was easily obtained when *in ovulo* and *in ovary* embryo culture were used after intraspecific crosses, even when proembryos were cultured. The culture medium used was simple and contained no growth regulators. Ovules cultured attached to placental tissues resulted in seed development when *in ovulo* embryo culture was used. When *in ovary* embryo culture was used, fruit and seed development occurred only when the fruit was cut longitudinally or when the basal portion of it was removed previous to plating.

In vitro pollination techniques failed because, in spite of good pollen germination and tube growth, pollen tubes failed to penetrate the ovarian cavity, both in intra- and interspecific pollinations.

SECTION I

INTRODUCTION

The genus *Vaccinium* comprises approximately 130 species, 24 of which are native to eastern North America (CAMP, 1945). Some species produce edible berries that have been harvested and consumed by man for centuries. Commercial domestication of blueberries in North America began in north Florida in 1893 when native rabbiteye blueberry bushes (*Vaccinium ashei* Reade) were transplanted from the woods and swamps to establish the first commercial plantations (MOWRY and CAMP, 1928). Although almost 40 % of the United States blueberry production still comes from wild stands, there are now more than 10,000 ha planted with improved cultivars (CHILDERS, 1983).

Cultivar improvement was initiated in 1906 by Frederic V. Coville, who in 1920 introduced for New Jersey the first 3 commercial cultivars of the highbush type. Since then, improvement programs have been started in several other areas of the United States and Canada under widely differing edaphic and climatic conditions (GALLETTA, 1975). The continuous release since 1920 of improved cultivars adapted to particular growing conditions has resulted in the development of a strong industry in the United States and in an increased interest in blueberry growing in other areas of the world (CHILDERS, 1983).

Taxonomy of blueberries has been treated variously (CAMP, 1945; AALDERS, 1962; WARD, 1974; VANDER KLOET, 1980), but Camp's (1945) monograph is the most extensive and has gained wide acceptance among blueberry breeders. According to Camp, cultivated blueberries belong to the family *Ericaceae*, subfamily *Vacciniaceae*, genus *Vaccinium*, subgenus *Cyanococcus*, the cluster-fruited blueberries. The basic chromosome number of *Vaccinium* is considered to be x=12. Polyploidization has played a major role in speciation of the genus, and diploid (2x=24), tetraploid (4x=48), and hexaploid (6x=72) species are found among native North American blueberries (DARROW et al., 1944).

Interspecific hybridization has been very important in the improvement of blueberries (GALLETTA, 1975; MOORE, 1966), but most interspecific hybrids used to obtain improved cultivars have come from crosses between species with the same chromosome number (COVILLE, 1937; GALLETTA, 1975; MOORE, 1966). Heteroploid crosses are difficult to make, and in most cases hybrids are rarely obtained (COVILLE, 1937; DARROW and CAMP, 1945; SHARPE and SHERMAN, 1971). Despite this fact, the few hybrids obtained so far from heteroploid crosses have been extremely useful in broadening the adaptability of this fruit crop.

Although a number of species have been used in blueberry improvement, there remain still other species, at all 3 ploidy levels, that possess traits of horticultural importance and therefore could be used in breeding cultivars for particular conditions (MOORE, 1966). *V. elliottii* Chapm. is one of the species with potential for improving cultivars adapted to north-central Florida (LYRENE and SHERMAN, 1977). It is a small-fruited, wild, diploid species abundant in suitable habitats in northern Florida and in much of the rest of the southeastern

United States. Introgression of genes from this species into improved tetraploid cultivars of *V. corymbosum* L. would enable breeders to bring together the high fruit quality and high yields of the tetraploid cultivars with the early ripening, tolerance for dry upland soils, disease resistance, and delightful fruit flavor and aroma of the best *V. elliottii* selections (LYRENE and SHERMAN, 1977). Furthermore, if triploid hybrids could be obtained from this cross, a synthetic hexaploid could be produced using colchicine. This synthetic hexaploid could then be crossed to hexaploid rabbiteye cultivars to produce a gene pool that would combine traits of diploid, tetraploid, and hexaploid species.

Attempts to produce hybrids when crossing *V. elliottii* with *V. corymbosum* usually fail, but occasionally some hybrids, with varying chromosome numbers, are obtained (LYRENE and SHERMAN, 1983). Since it would be highly desirable to produce a relatively large number of these hybrids, research was conducted to study the factors that reduce the number of hybrids obtained in crosses between these 2 species and to evaluate several *in vitro* techniques that might enhance hybrid production.

SECTION II

LITERATURE REVIEW

Interspecific Crosses in Blueberry

Interspecific hybridization has been used by plant breeders mainly to incorporate known useful characters that are unavailable in the species being improved but are present in closely related wild species. Other reasons for making interspecific crosses include the exploitation of interspecific heterosis, the development of new genetic combinations to permit the expression of latent genes, the broadening of the genetic base of the breeding population, the study of taxonomic and phylogenetic relationships among species, and to provide a bridge between incompatible species (FEHR and HADLEY, 1980; UHLINGER, 1982; LAYNE, 1983).

Interest in interspecific hybridization began early in the history of blueberry improvement. The first highbush cultivars released for New Jersey growers were first-generation hybrids between selections of *Vaccinium corymbosum* L. and *V. australe* Small (COVILLE, 1937). Lowbush species, mainly *V. angustifolium* Ait., were used later to develop highbush cultivars for Michigan and other eastern United States locations (MOORE, 1966). Some cultivars released in West

Virginia came from crosses between *V. pallidum* Ait., a diploid species, and 'Concord', a *V. corymbosum* X *V. australe* hybrid (CHILDS, 1969). Cultivars released for Florida are mainly derived from crosses between *V. corymbosum* and *V. darrowi* Camp; however, *V. tenellum* Ait., *V. angustifolium*, and *V. ashei* Reade are also represented in the breeding population (SHARPE and SHERMAN, 1971).

Darrow and Camp (1945) were the first to systematically study crossability among various *Vaccinium* species. They confirmed what Coville (1937) had already noticed and is now a well-known fact among blueberry breeders: within subgenus *Cyanococcus*, crosses among species of the same chromosome number are easy to make, but crosses between species of different chromosome number are more difficult (MOORE, 1966; GALLETTA, 1975).

Attempts to make heteroploid crosses have resulted in various degrees of success, depending on the species used, their ploidy level, and the particular genotypes involved in the cross. Hybrids between hexaploid and diploid species can be obtained with great difficulty. Sharpe and Darrow (1959) reported that an average of 1,500 pollinations were needed to obtain 1 true hybrid when crossing *V. ashei* (6x) with *V. darrowi* (2x). Previously, Darrow et al. (1954) had also obtained tetraploid hybrids using these same species as parents, and when crossing *V. ashei* with other diploid species such as *V. atrococcum* Heller and *V. tenellum*. Draper (1977) also obtained a tetraploid hybrid when crossing a hexaploid hybrid (*V. ashei* X *V. constablaei*) with *V. darrowi*.

Pentaploids are readily produced by crossing hexaploid and tetraploid species (DARROW and CAMP, 1945; JELENKOVIC and DRAPER, 1973).

These hybrids, however, have limited use in breeding because they generally exhibit various degrees of sterility (DARROW et al., 1952), and because their backcross derivatives appear less vigorous than the original pentaploid hybrid (HASSAN, 1983).

Despite the fact that one natural triploid clone has been reported (AHOKAS,1971), triploids are extemely difficult to obtain when crossing diploid X tetraploid plants. Instead, tetraploid hybrids are sometimes obtained (SHARPE and SHERMAN, 1971; BALLINGTON and GALLETTA, 1976). Darrow and Camp (1945) obtained few seedlings when crossing *V. australe* (4x) with *V. darrowi* (2x), but they did not report on their ploidy. Draper (1977) was able to produce tetraploid hybrids when crossing *V. corymbosum* (4x) X *V. darrowi* (2x) and when crossing an interspecific diploid hybrid (*V. darrowi* X *V. atrococcum*) with *V. corymbosum* (4x). Sharpe and Darrow (1959) found that the cross of *V. corymbosum* (4x) and *V. darrowi* (2x) produced tetraploid hybrids readily, since an average of only 52 pollinations was required to obtain one hybrid seedling. Childs (1969) was also successful in producing tetraploids when crossing the hybrid *V. corymbosum* X *V. australe* (4x) with *V. pallidum* (2x). Lyrene and Sherman (1983) produced 25 hybrids after pollinating about 7,000 flowers of *V. corymbosum* (4x) with pollen of *V. elliottii* Chapm. (2x). The resulting hybrids included tetraploids, pentaploids, aneuploids and a few triploids.

The production of tetraploid hybrids in crosses between diploid and tetraploid blueberries results from the ability of various diploid species to produce functional 2n gametes (SHARPE and SHERMAN, 1971; BALLINGTON and GALLETTA, 1976; LYRENE and SHERMAN, 1983).

Tetraploids can also produce functional 2n gametes in crosses with diploid species, yielding pentaploid hybrids (LYRENE and SHERMAN, 1983).

Some crosses between diploid and tetraploid species have been consistently unsuccessful. This is the case of the cross between *V. angustifolium* (4x) and *V. myrtilloides* Michaux (2x) which, although attempted several times, has not yielded any hybrids (LONGLEY, 1927; COVILLE, 1937; DARROW and CAMP, 1945; AALDERS and HALL, 1961). Probably, several other crosses have been equally unsuccessful and therefore are not mentioned in the literature.

The rarety or complete absence of triploids from diploid X tetraploid crosses is not unique to blueberries, and has been reported for many different plant genera (WOODELL and VALENTINE, 1961). Marks (1966) first referred to this phenomenon as the "triploid block," terminology that is now used frequently in the literature.

Several different mechanisms may prevent the formation of hybrid zygotes in interspecific crosses. These mechanisms establish upper limits to outbreeding and panmixis, contributing in nature to the isolation of populations. This favors speciation and a gradual increase in polymorphism within genera and families (STEBBINS, 1958; DE NETTANCOURT, 1977).

Causes of Interspecific Incompatibility

A variety of incompatibility barriers can prevent the formation of interspecific hybrid zygotes, prevent their development after fertilization, or drastically reduce the fertility of the resulting hybrids.

Prefertilization Barriers

Prefertilization barriers can be morphological, physical, or can result from a cell-to-cell recognition event mediated by particular molecules. Differences in flower morphology can result in cross-incompatibility. For instance, differences in length between styles and pollen tubes may prevent fertilization (KHO and BAER, 1973), or the presence of pubescence may prevent pollen from reaching the stigmatic surface, thus impeding germination (IBRAHIM and COYNE, 1975).

In cases where there are no morphological differences between the flowers of the parents, pollen rejection may result from simple chemical or physical reasons. Phenolic compounds have been shown to selectively promote or inhibit pollen germination (MARTIN and RUBERTE, 1972). Also, the lack of a specific substance at the pollen-stigma interphase may impede pollen hydration or pollen tube penetration of the stigmatic cuticle (KNOX et al., 1976).

Based on these observations, which indicate that the rejection process is passive, Hogenboom (1975) proposed that interspecific incompatibility, which he terms "incongruity," is a phenomenon completely distinct from self-incompatibility. According to this author, incongruity is due to the lack of genetic information in one partner about some relevant character of the other, which results in the inability of the pollen to germinate or in the inability of the pollen tube to grow down the style.

However, there is considerable evidence suggesting that, at least in closely related species, interspecific incompatibility is a function of the same S-gene complex controlling self-incompatibility

(LEWIS and CROWE, 1958; ABDALLA and HERMSEN, 1972; PANDEY, 1980, 1981) and therefore is an active process mediated by proteins and glycoproteins (HOWLETT, et el., 1981). Many of these proteins and glycoproteins show antigenic properties and lectin-binding sites, suggesting that they are involved in a molecular recognition similar to that found in animals (BATES and DEYOE, 1973; MARCHALONIS, 1976).

Interspecific incompatibility is probably under sporophytic control in those plant families where self-incompatibility is governed sporophytically (DE NETTANCOURT, 1977). In the sporophytic system, pollen reaction is determined by the maternal diploid genome and the rejection occurs at the stigmatic surface. Sporophytic self-incompatibility is characteristic of the *Cruciferae, Compositae,* and *Rubiaceae,* which all have trinucleate pollen. (LEWIS, 1979).

Similarly, in plant families with gametophytic self-incompatibility, interspecific incompatibility is probably under gametophytic control. Pollen reaction in the gametophytic system is determined by its own hapolid allelic constitution and the rejection occurs in the style. Gamatophytic self-incompatibility is characteristic of the *Rosaseae, Leguminosae, Onagraceae, Scrophulariaceae, Solanaceae, Ranunculaceae,* and *Chenopodiaceae,* which all have binucleate pollen and wet stigmas (LEWIS, 1979).

The most remarkable feature of interspecific incompatibility in closely related species is that it usually occurs unilaterally, preventing self-incompatible species from accepting pollen of species which reproduce through selfing (LEWIS and CROWE, 1958). By contrast, the reciprocal cross does not show hybrid incompatibility. This

phenomenon is generally referred to as "unilateral incompatibility" and is of common occurence in various plant genera among several families (PANDEY, 1981).

Characterization of the incompatible reaction and studies of the biochemical nature of the incompatibility substances are abundant for self-incompatibility systems (KNOX, et al., 1976; ROBERTS et al., 1980; HESLOP-HARRISON, 1982). However, comparatively few studies have been done to observe the features of the rejection process after incompatible pollinations between different species.

Although evidence was not universal, Lewis and Crowe (1958), reviewing observations in various genera, concluded that in general the interspecific incompatibility reaction occurred earlier than the self-incompatibility reaction, but at the same sites as in sporophytic and gametophytic self-incompatability. In some species, however, the rejection reaction does not occur at the stigma or style, but inside the ovary. This situation occurs in crosses between *Panicum coloratum* and *P. antidotale*. In this cross, after pollen tubes reach the ovary they become disoriented, grow in a random fashion and fail to penetrate the micropyle (BURSON and YOUNG, 1983). A similar situation has been described in crosses of *Rhododendron impeditum* and *R. williamsianum* (KHO and BAER, 1970).

Studies done in *Populus* with fluorescence and scanning electron microscopy have demonstrated that when rejection occurs in the style, pollen tubes show a progressive retardation of growth and a twisted appearance (STETTLER et al., 1980) with heavy callose deposition (WILLING and PRYOR, 1976). In *Rhododendron*, 10 different abnormalities in pollen tube growth along the stylar canal have been detected after

interspecific crossing. They include bursting, coiling, and spiralling of the tubes, and tubes with variable diameters, tapered tips, or with abnormal callose depositions (WILLIAMS et al., 1982).

The only ultrastructural study of interspecific incompatibility has been done in crosses between species of *Lycopersicon* (DE NETTANCOURT, 1977). The rejection reaction during early pollen tube growth is similar to the rejection reaction during selfing. It is characterized by the appearance of concentric endoplasmic reticulum in the cytoplasm and by a progressive disappearence of the callose-rich inner cell wall. This is associated with an accumulation of electron dense particles of unknown origin in the cytoplasm. At later stages, however, the interspecific incompatibility reaction differs from the self-incompatibility reaction in that there is a progressive breakdown of the outer wall, resulting in its disaggregation into a great number of loose particles of irregular shape dispersed in the intercellular spaces of the stylar tissues. Simultaneously, the callosic plug at the tip of the tube gradually disappears and the tip opens, allowing the tube contents to flow out between the stylar cells.

Self-incompatibility in the *Ericaceae* is considered to be of the gametophytic type (PANDEY, 1960). In *Vaccinium*, different degrees of self-incompatibility occur among species at all 3 ploidy levels. Futhermore, various degrees of self-compatibility exist among clones within species (MEADER and DARROW, 1944; EL-AGAMY et al., 1981). Cultivated species range in their degree of self-compatibility from the almost self-compatible highbush to the highly self-incompatible rabbiteye (EL-AGAMY et al., 1981). Lowbush blueberries also exhibit a high degree of self-incompatibilty (AALDERS and HALL, 1961; WOOD, 1965)

and, according to Ballington (cited by GALLETTA, 1975), most diploid species are also largely self-incompatible.

Unilateral incompatiblity has not been found in blueberry. In fact, according to El-Agamy et al. (1979), pollen germinates and grows to the base of the style in all heteroploid crosses irrespective of the direction of the cross. *V. darrowi* (2x), *V. corymbosum* 'Sharpblue' (4x), and *V. ashei* 'Aliceblue', 'Beckyblue' and 'Climax' (6x) were used by El-Agamy et al. in their studies.

Postfertilization Barriers

A number of different mechanisms prevent or retard the development of hybrids from the first division of the zygote up to the final differentiation of the reproductive organs. Stebbins (1958) recognized 3 major mechanisms involved in postfertiization incompatibility: a) incompatibility between parental chromosomes or genes; b) incompatibility involving cytoplasmic and plastid differences; and c) incompatibility between embryo and endosperm. This last type of incompatibility, which has been also called "ploidy barrier to crossing," is the one that prevents the formation of triploids in crosses of diploids X tetraploids and will be considered in detail in the following paragraphs.

Genome incompatibility is clearly not the problem in crosses between a diploid and its induced autotetraploid, but triploids are very rarely obtained when making such crosses. Woodell and Valentine (1961) surveyed the results of a series of crosses between diploids and their induced autotetraploids and found that there was much variation in the

expression of the triploid block, but those crosses producing an abundance of triploids were uncommon.

Incompatibility may be genic in hybrids between an allopolyploid and one of its parental diploids, but there are a number of cases where genic incompatibility does not occur. For instance, when crossing the tetraploid *Lamium intermedium* with 2 of its diploid ancestors, *L. purpureum* and *L. amplexicaule*, no seed is obtained regardless of the direction of the cross. However, the artificially produced autotetraploid of both *L. purpureum* and *L. amplexicaule* produce hybrids rather easily with *L. intermedium*, demonstrating that in this case genic differences are not the cause of incompatibility. The same was demonstarted in *Vaccinium* by Draper el al. (1972) working with *V. atrococcum* (2x) and *V. corymbosum* (4x). This cross is completely unsuccessful, but if an autotetraploid is produced by doubling the chromosome number of the diploid species, the autotetraploid produced crosses readily with *V. corymbosum*.

A characteristic common in heteroploid crosses is the occurrence of marked reciprocal differences, which can not be attributed to cytoplasmic differences (DHALIWAL, 1977). It has been found that the cross is generally more successful when the higher ploidy level species is used as female parent (WOODELL and VALENTINE, 1961). Gill and Waines (1978) found that in *Triticum* crossability is under paternal control. They showed that a factor present in the pollen, which shows dosage effects, leads to embryo abortion by interacting with the maternal genome.

In most heteroploid crosses where an incompatibility reaction prevents the production of hybrids, the endosperm shows abnormal

behavior leading to the early abortion of the embryo. Several hypotheses have been proposed to explain the failure of the endosperm in heteroploid crosses.

In the early 1930's Muntzing (1933), working with crosses between diploid *Galeopsis* species and their induced autotetraploids, proposed that for a successful cross the number of chromosome sets should be in a particular ratio among the 3 tissues involved in seed development. Since the genomic ratio of maternal, endosperm, and embryo tissue for the resultant seed was either 4:5:3 or 2:4:3 in each reciprocal cross of a diploid x a tetraploid, he concluded that the principal reason for poor seed development was a deviation from the normal 2:3:2 ratio characteristic of successful homoploid crosses.

Watkins (1932) found some exceptions to the Muntzing hypothesis while working with interspecific hybrids of *Digitalis* and with the intergeneric hybrid, *Raphanobrassica*. He found some cases in which maternal:endosperm:embryo ratios of 2:6:4 produced viable seeds. He concluded that a quantitative change in the relations between seed components was important, but that only the endosperm and embryo should be considered.

Stephens (1942), trying to explain crossability between different homo- and heteroploid crosses in *Gossypium*, proposed that for best results, the embryo:endosperm ratio should be 2:3, but that differences in compatibility are associated with quantitative rather than qualitative differences in the cytological balance between endosperm and zygote. These differences give rise to species with different genomic "strength" within a given ploidy level which produce fertile crosses by behaving as if they were of a higher ploidy level.

Valentine (1956) proposed this same "strength" hypothesis, to meet the 2:3 maternal tissue : endosperm ratio criterion in *Primula*.

Kihara and Nishiyama (1932), studying interspecific crosses in *Avena*, were the first to propose that only the genetic constitution of the endosperm is important. According to their view, endosperm development is conditioned by the balance in strength between the paternal and maternal chromosomes. Chromosomes contributed by the male in excess of the regular constitution of the female will result in an imbalance leading to endosperm degeneration.

This concept was developed by Nishiyama (NISHIYAMA and INOMATA, 1966; NISHIYAMA and YABUNO, 1978) in what he called the "polar nuclei activation" hypothesis, in which the strength of the activating action of the male nucleus is expressed in terms of activating value (AV) and response value (RV) respectively. The degree of seed failure is closely related to the difference between the activating and response values, expressed by the activating index (AI)=(AV/2RV) of the polar nuclei. The AI is 0.5 (or 50%) in a selfed plant and normal seeds usually result. If the AI deviates from 0.5, the endosperm does not develop and the embryo aborts. In other words, they suggested that successful endosperm development depends on a 1:2 ratio of paternal:maternal genomes in the endosperm. This same relationship was demonstrated by Lin (1975) in maize. He also demonstrated that for normal seed development, the endosperm may be of any ploidy level multiple of 3x, provided that paternal and maternal genomes are in a 1:2 ratio.

This same reasoning was used by Johnston et al. (1980) to elaborate their "endosperm balance number" (EBN) hypothesis. According

to this hypothesis the genome of each species is assigned an EBN on the basis of its crossing behavior to a standard species. The EBN determines the effective ploidy in the endosperm of each species and must be in a 1:2 paternal:maternal ratio. Usually each genome is assigned one EBN in a way such that for monoploids EBN=1, for diploids EBN=2, for tetraploids EBN=4, and so on. In some species, however, the EBN is not the direct reflection of its ploidy, so that these species cross as if they were of a different ploidy level. These authors regard the EBN as a single gene rather than a factor that involves the whole genome.

Anatomical and histological studies have demostrated that endosperm breakdown is often associated with embryo abortion. Although a causal relationship between endosperm and embryo abortion has sometimes been difficult to establish, the observation has been made that, endosperm breakdown usually precedes embryo abortion (STEBBINS, 1958; RAGHAVAN, 1976). This is not a universal phenomenon. In heteroploid crosses in *Citrus*, many embryoless seeds have normal endosperm, while in seeds that contain an embryo, the endosperm invariably degenerates (ESEN and SOOST, 1973), suggesting that in this case the presence of one is not essential for the development of the other. Similarly, embryo degeneration in *Lycopersicon* may commence when the endosperm is still healthy (COOPER and BRINK, 1945) and embryo abortion in *Trifolium* always precedes endosperm disintegration in some species (KAZIMIERSKA, 1980), but the opposite is true in others (WHITE and WILLIAMS, 1976).

Aalders and Hall (1961) found that in blueberry, crosses between *V. myrtilloides* (2x) and *V. angustifolium* (4x), fertilization

occurred normally and the endosperm formed 6 to 8 nuclei before it started to disintegrate. At this stage, the zygote is usually undivided in blueberries (STUSHNOFF and PALSER, 1969).

The morphology of embryo and endosperm breakdown is well documented in some species. For example in *Lycopersicon*, endosperm breakdown was preceded by a slowdown in the rate of cell division and by an increase in cell vacuolation. Once cell division ceased, cell walls disappeared and one or a few giantic polynucleate cells were formed that completely surrounded the embryo (COOPER and BRINK, 1945; BARBANO and TOPOLESKI, 1984). Enlargement of cells followed by the formation of pycnotic nuclei and granular cytoplasm has been observed in disintegrating endosperm cells of *Datura* (RIETSEMA and SATINA, 1959). In *Medicago*, endosperm failed to form cell walls, or cell wall formation was retarded to various degrees (LENDINGHAM, 1940).

In most species, endosperm breakdown is preceded by mitotic abnormalities such as chromosome breakage (STEBBINS, 1958) and lagging chromosomes which lead to the formation of aneuploid nuclei (RAGHAVAN, 1976).

Stebbins (1958), in his extensive review of the causes of hybrid inviability in crosses between diploids and tetraploids of 6 different species, concluded that in 2 of the 6 cases, there was no difference either in the degree or nature of endosperm development regardless of the direction of the cross. In all other combinations, endosperm developed precociously in 2x x 4x crosses, and was retarded in 4x x 2x crosses, as compared to normal homoploid crosses.

Abortion of the embryo can occur between zygote formation and the cotyledonary stage of embryo development, but in most cases

involving the triploid block, embryos will never develop beyond a few cells (RAGHAVAN, 1976). The first zygotic division in *Datura* is usually delayed, and subsequent divisions result in formation of a completely undifferentiated group of cells or in formation of differentiated misshapen embryos. These abnormal embryos may possess cells that are devoid of content or suspensors that are unusually long, short, or broad (RIETSEMA and SATINA, 1959).

Ashley (1972), crossing 2x and 4x *Hibiscus*, showed that the shrinkage of the zygote that usually follows fertilization in viable crosses did not occur in inviable ones, nor did polarization of the zygotic cell take place. Instead, there was a large decrease in the number of organelles present and in the density of the cytoplasm, followed by a marked increase in cell vacuolation. As a result, hybrid embryos formed no more than a clump of highly vacuolated necrotic cells that aborted by the time the normal embryo had reached the globular stage.

When abortion occurs late during seed development, as in *Gossypium* (PUNDIR, 1972), branching of the cotyledons, differentiation of multiple shoots from them, and other aberrations are observed in hybrid embryos. In this same genus, abortion occurs in some cases during seedling development. Ultrastructural studies of cell necrosis in the seedling lethals show that the first subcellular abnormality is the degeneration of the inner membrane and cristae of the mitochondria (PHILLIPS and REID, 1975).

It has been proposed that in some species, tissues other than the endosperm are involved in embryo abortion. For example, the antipodals degenerate prematurely in *Hordeum* x *Secale* crosses (BRINK and

COOPER, 1944). In certain infertile hybrids abnormalities also occur in somatic tissues. The term "somatoplastic sterility" was first used by Brink and Cooper (1947) to describe seed collapse associated with a retardation in growth of the endosperm produced by a pronounced hyperplastic growth of the nucellus in *Nicotiana* and *Medicago*. In *Datura* (RIETSEMA and SATINA, 1959) and in *Lycopersicon* (COOPER and BRINK, 1945), abortion resulted from tumors formed at the inner epidermis of the integument, the endothelium, which grew into the embryo sac forming a voluminous mass of tissue that completely surrounded the embryo, preventing its contact with the endosperm. These endothelium tumors produced a water-soluble, heat stable substance which inhibited *in vitro* growth of normal embryos (SATINA et al., 1950).

The underlying physiological causes of embryo and endosperm malfunction are poorly understood. The fact that endosperm breakdown precedes embryo abortion in some species and that hybrid seedlings can be obtained by extracting and culturing the hybrid embryo *in vitro*, raises the possibility that failure of the hybrid endosperm to supply specific growth factors to the embryo is the cause of abortion. This has been considered a possibility in hybrids between *Phaseolus vulagris* X *P. acutifolius*, where greatly reduced levels of cytokinin-like compounds were found in hybrid ovules as compared to non-hybrid ones (NESLING and MORRIS, 1979). Histochemical studies in *Medicago* (SANGDUEN el al., 1983) have demostrated that, in inviable hybrid embryos, starch and lipids are not metabolized out of the maternal tissue between 5 and 7 days after pollination as in the case of viable embryos. Later, starch and lipids are not mobilized out of the endosperm and nucellus. At the heart stage of embryo developement,

relative inactivity of dictyosomes and endoplasmic reticulum in the nucellus, integumentary tapetum, endosperm, and suspensor, is indicative of a failure in nutrient metabolism and transport leading to embryo abortion.

Overcoming Interspecific Incompatibility

Different methods have been developed and successfully applied to overcome the various incompatibility barriers that prevent the production of interspecific hybrids.

Prefertilization Barriers

Prefertilization barriers can be overcome by manipulating pollen-pistil interactions mechanically, physically, or chemically in order to alter their recognition potential. The hypothesis that mechanisms controlling interspecific incompatibility are the same as those controlling self-incompatibility has already been mentioned (PANDEY, 1981). If this hypothesis is correct, methods successful in overcoming self-incompatibility should be equally successful in overcoming interspecific incompatibility. This is not the case, however, since only a few methods have been successful for overcoming interspecific barriers. Methods to overcome self-incompatibility have been reviewed elsewhere (DE NETTANCOURT, 1977; FRANKEL and GALUM, 1977; SHIVANNA, 1932). Techniques used include induced mutations, induced autopolyploidy, use of irradiation (X-rays, UV-rays), bud pollination,

delayed pollination, hot water and high temperature treatments, use of organic solvents, increased atmospheric humidity, grafting and mutilation of stigma and style, application of growth regulators, application of electrical charges, use of mentor pollen, and *in vitro* pollination. The failure of some of these methods to overcome incompatibility is probably because, in most species, loci different from the S-locus are controlling incompatibility or other mechanisms are superimposed over the S-gene system (DE NETTANCOURT, 1977).

Several methods have been successfully used to overcome prefertilization barriers in interspecific crosses. Mechanical methods were probably the first to be used. Most of these methods involve style amputation or style transplants. Using such techniques, Mangelsdorf and Reevs (cited by CLARKE and KNOX, 1978), as early as 1930, produced hybrids between *Zea* and *Tripsacum*. Later on, interspecific hybrids were produced among different species of *Lathyrus* (DAVIES, 1957) and *Solanum* (SWAMINATHAN, 1955).

A more sophisticated approach is to completely bypass the style and stigma pathway by directly injecting a pollen suspension into the ovary. This technique, which has been called "intraovarian pollination", was successfully used to produce interspecific hybrids in *Argemone* (KANTA and MAHESHWARI, 1963) and a few other species (ZENKTELER, 1980). There are, however, many limitations in extending this technique to other taxa (SHIVANNA, 1982).

The use of radiation to induce mutations has had variable success. In cases where favorable mutations have been induced, it is not clear if the lesion was produced at the S-locus or at other loci

controlling other aspects of the physiology of pollen grains (DE NETTANCOURT, 1977).

Growth regulators, particularly auxins, applied to the flower immediately after pollination can increase the production of interspecific hybrids in *Lilium* (EMSWELLER and STUART, 1948) and *Vaccinium* (DARROW, 1956).

The increasing knowledge of the biochemical nature of the incompatibility reaction has opened new avenues for overcoming interspecific incompatibility, mainly through the use of chemical treatments. Probably the most elegant experiments with chemical treatments are those of Willing and Pryor (1976) with *Populus*. They showed that by washing the stigma or pollen with n-hexane or ethyl acetate, the putative incompatibility receptor can be removed so that the incompatibility system is no longer effective. Earlier they showed that by extracting compatible pollen with ethyl acetate and other anhydrous solvents, and coating the incompatible pollen with this solvent-extracted solution, the incompatibility barrier can be broken so that the stigma will no longer reject the foreign pollen (KNOX et al., 1972; WILLING and PRYOR, 1976).

This same basic principle also probably underlies the so-called "mentor pollen technique," which has been successfully used in plant breeding for more than 30 years. A mixture of compatible and incompatible pollen is used when making the cross. Compatible pollen must be inactivated by using a high dose of irradiation, a chemical treatment, or repeated freezing and thawing (PANDEY, 1979). The list of taxa where this technique has worked is quite extensive (PANDEY, 1979).

To test the hypothesis that crossability barriers result from a specific immunochemical reaction, Taira and Larter (1977) injected immunosuppressive compounds, such as e-amino-n-caproic acid or its analog L-lysine-HCl, to flowers before pollination. Using this technique, they were able to increase the rate at which triticale hybrids (*Triticum* X *Secale*) can be obtained.

More recently, *in vitro* pollinations have been used to overcome interspecific incompatibilities. Ovules or ovules attached to placental tissues are extracted from flowers before anthesis and placed in a nutrient medium. Pollen grains are subsequently put directly on the surface of the ovules or in close proximity to them in order to bring about fertilization. Although fertilization does occur when using this method, very few cases are reported where hybrids have been raised to maturity (ZENKTELER, 1980).

Postfertilization Barriers

It has been already mentioned that embryo abortion in some cases results from an inadequate development of the endosperm. Laibach (cited by RAGHAVAN, 1976), as early as 1925, found that in the cross between *Linum austriacum* and *L. perenne*, viable seeds were never formed, but he was able to produce hybrid plants by dissecting the embryos from the seed and culturing then *in vitro*.

Since the early work of Laibach, who used a very simple medium to culture the embryos, several inviable crosses have been made possible using increasingly complex culture media and various refined techniques. Raghavan (1977) listed more than 40 cases where embryo

culture has been used in trying to overcome interspecific incompatibilities, many of them in important agricultural genera.

Comprehensive reviews of the methods used for embryo culture can be found elsewhere (RAPPAPORT, 1954; SANDERS and ZIEBUR, 1963; MONNIER, 1978; NORSTOG, 1979; RAGHAVAN, 1976, 1977, 1980; RAGHAVAN and SRIVASTAVA, 1982). It was mentioned that embryo abortion in interspecific crosses usually occurs during the proembryonic stages of seed development, i.e. before the cotyledons differentiate in the embryo. Proembryos are difficult to culture because they need very specific nutritional and cutural conditions (RAGHAVAN, 1976, 1977, 1980). Furthermore, they are difficult to dissect out of the ovules without wounding the suspensor, which is particularly sensitive. Damage to the suspensor is thought to be the main cause of failure when culturing proembryos (MONNIER, 1978).

There is much evidence to demostrate that the nutritional requirement of the embryo is highly specific, particularly during its early development (MAHESHWARI, 1950). The contention has been made that in interspecific crosses some of these specific nutrients are not produced by the endosperm, and that lack of these nutrients prevents the embryo from developing (RAGHAVAN, 1976). Consequently, a variety of substances have been added to the nutrient media in efforts to find the missing compound.

A breakthrough in the culture of proembryos was obtained by Van Overbeek et al. (1942) when they added coconut milk to a highly complex medium and found that embryo growth could be dramatically increased. Since Overbeek's discovery, extracts of several other endosperms and seeds have been tried, often with inconsistent results

(RAGHAVAN and SRIVASTAVA, 1982). Also, several attempts have been made to define the exact composition of coconut and other endosperms. Norstog (1979), studying the result of several endosperm analyses, found that citrate and malate were present in high concentrations in most endosperms. He recommended the inclusion of these 2 components in the nutrient media, particularly in the form of an ammonium salt, since ammonium ions have been shown to enhance embryo growth in culture compared to other nitrogenous sources. Claims have also been made that myo-inositol and sorbitol (NORSTOG and SMITH, 1963), which are also found in coconut milk, can be used advantageously for embryo culture.

Effects of growth regulators are highly inconsistent and rather contradictory, but generally a balanced mixture of auxins and cytokinins is required (RAGHAVAN, 1980; RAGHAVAN and SRIVASTAVA, 1982). Addition of particular growth regulators is sometimes essential in culturing embryos of some species. For instance, picloram, a synthetic auxin, is required for *Trifolium* embryo culture (PHILLIPS, 1981).

The observation that the liquid endosperm which bathes proembryos has a high osmotic value (SMITH, 1973) led to the thought that the osmoticum of the nutrient medium is important in the culture of proembryos. This is probably not true for all species, since although sucrose concentrations of up to 18 % are needed in some species, relatively low concentrations (2-3 %) are sufficient for others (RAGHAVAN, 1976; RAGHAVAN and SRIVASTAVA, 1982).

In some cases the hybrid zygote can produce an undifferentiated callus, that when placed under appropriate culture conditions becomes highly embryogenic. This "embryo callus" technique

has been used by Thomas and Pratt (1981) to produce interspecific hybrids in *Lycopersicon*.

Embryo abortion sometimes occurs very early during seed development, at a stage when proembryos are almost impossible to dissect out of the ovule. In those cases, treatments to delay embryo abortion have been used previous to the culture of the hybrid embryos. A mixture of gibberellic acid, naphthaleneacetic acid, and kinetin, applied to recently pollinated flowers, was used by Gosal and Bajaj (1983) to delay embryo abortion in crosses with *Vigna*.

In ovulo embryo culture has been considered as another alternative to embryo culture in those cases where embryos are difficult to dissect or where abortion occurs at the proembryonic stages of seed development. Attempts to culture proembryos without removing them from the ovules started in 1958 when Maheshwari showed that fertilized eggs or two-celled proembryos of *Papaver somniferum* could be grown to maturity in culture (MAHESHWARI, 1958). This appeared quite significant since it is extremely difficult to culture isolated embryos excised at this early stage of development. This technique has been successfully used to produce interspecific crosses in *Gossypium* (STEWART and HSU, 1978) and *Nicotiana* (REED AND COLLINS, 1978). More recently, interspecific hybrids were produced in *Impatiens* (ARISUMI, 1980) and in *Brassica* and *Raphanus* (TAKESHITA et al., 1980).

Sophisticated techniques have been developed for the culture of isolated embryos (MONNIER, 1978; WILLIAMS and DE LATOUR, 1980) in an effort to simulate the physical and nutritional conditions under which embryos normally develop. Such techniques are not required when using *in ovulo* embryo culture, since the embryo develops inside the ovule,

where conditions are more likely to be similar to those found *in vivo*. As a result, several relatively simple media have proven effective in *in ovulo* embryo culture (REED and COLLINS, 1978; STEWART and HSU, 1978; TAKESHITA et al., 1980).

Various growth regulators and other nutrient components, such as casein hydrolysate and coconut milk, have been added to the media to study their effect on embryo and ovule growth and development. Results indicate that in most cases they are not essential. Some of them, however, have a marked effect on growth rate of both embryos and ovules (RAGHAVAN, 1976; RANGAN, 1982).

Unexpectedly, the osmotic concentration of the medium seems also to be important for *in ovulo* embryo growth (RANGAN, 1982). Concentrations of 6-8 % are considered optimum for *in ovulo* embryo growth of *Petunia* (WAKIZUKA and NIKAJIMA, 1974) and *Prunus* (RAMMING, 1982). A high osmoticum is not essential, however, for *in ovulo* embryo culture of other species, where concentrations of only 3-4 % gave satisfactory results (REED and COLLINS, 1978; STEWART and HSU, 1978; TAKESHITA et al., 1980).

Factors other than the nutrient composition of the growing media appear to be more critical for ovule culture. For instance, it has been shown that in most cases ovules grow best when they are cultured attached to placental tissues (RAGHAVAN, 1976; RANGAN, 1982). The observation has also been made that placentas with many ovules are usually easier to culture than those with a single ovule (TAKESHITA et al., 1980; ZENKTELER, 1980). Time of excision of the ovules is also important: the earlier during seed development they are excised from the

ovary, the more difficult it is to raise them to full maturity in culture (RANGAN, 1982).

Embryo rescue of wide hybrids has been extended further by trying to culture embryos without removing then from the ovary. Ovary culture has been used to produce interspecific hybrids among different species of *Brassica* (INOMATA, 1968; MATSUZAWA, 1978; TAKESHITA et al., 1980), *Raphanus* (TAKESHITA et al., 1980) and *Chrysanthemum* (WATANABE, 1977).

Most ovary culture studies have been done to study the physiology of fruit development. These studies have shown that very young ovaries can develop into mature fruit in culture, provided that auxins are added to the medium (NITSCH, 1951). Addition of other growth regulators or other media components are innocuous or can result in various degrees of increase in the rate of fruit growth or in the final size attained by the cultured fruit (RAGHAVAN, 1976; RANGAN, 1982). Inomata (1968, 1977, 1978a, 1978b, 1979) also came to nearly the same conclusion when trying to produce interspecific hybrids in *Brassica*. Addition of casein hydrolysate, coconut milk, yeast extract, and other media components was not essential for *in ovary* development of hybrid embryos.

Several other factors which influence the success of ovary culture have been noted. A remarkable condition usually found when unpollinated or recently pollinated flowers are put in culture is that they may develop into full size fruits, without developing any seeds or with only inviable seeds in them (NITSCH, 1951). This ability for parthenocarpic fruit development is species dependent (RANGAN, 1982). The presence of floral organs, particularly the calyx, is sometimes

essential for the development in culture of the ovary and the embryo inside it (RAGHAVAN, 1976; RANGAN, 1982). Finally, when the production of interspecific hybrids is the objective of ovary culture, it has been noted that the rate of hybrid production is highly dependent on the genotype used as female parent (INOMATA, 1978a).

SECTION III

CAUSES OF THE INTERSPECIFIC CROSS-INCOMPATIBILITY BETWEEN *Vaccinium corymbosum* L. AND *V. elliottii* CHAPM.

Introduction

A series of studies was conducted to determine the site of the incompatibility reaction in reciprocal crosses between *V. corymbosum* L. and *V. elliottii* Chapm. Pollen viability, pollen tube growth, Percent fertilization, and early embryo and endosperm development were studied.

Materials and Methods

During the flowering seasons of 1982 and 1983, pollinations were made using several cultivars and selections from the University of Florida blueberry breeding program. Tetraploid cultivars and selections are considered to be *V. corymbosum* although several other species are included in their genetic background (LYRENE and SHERMAN, 1983). *V. elliottii* plants were selected from native populations in Silverhill, Alabama (Baldwin County) and LaCrosse, Florida (Alachua County). Pollen of *V. elliottii* was also collected from 12 randomly

selected wild plants from LaCrosse and 3 plants from Baldwin, Florida (Nassau County). These 2 pollen mixtures were assigned the numbers FL 82-226 and FL 82-227 respectively.

Eight *V. elliottii* and 12 *V. corymbosum* selections were potted and placed in a cold chamber at 5°C in late fall to assure that their chilling requirements were met. This treatment resulted in a simultaneous bud-break in the spring and a profuse and uniform flowering. Plants were moved out of the cold chamber into a greenhouse, as they were needed, in groups of 4 or 6, starting in February and for a period of 3 months.

Both intra- and interspecific reciprocal crosses were made in the greenhouse. Before anthesis, flowers were prepared for pollination by removal of the corolla and careful emasculation to avoid self-pollination. Particular care was taken when *V. corymbosum* was used as the female parent, since this species is known to be partially self-compatible (EL AGAMY et al., 1981). After emasculation pollen was applied to the stigmas using the fingernail technique (GALLETTA, 1975).

For 1982 pollinations, a recently collected pollen mixture was used. During 1983, however, most pollinations were made using a mixture of stored pollen. For storing the pollen, flowers were collected, air dried, and placed in paper bags in a refrigerator at 2-4°C. Pollen stored under these conditions was used within 3 months, as needed.

Evaluation of Pollen Viability

Pollen viability was evaluated by *in vitro* germination and by observation of the pollen grains with a scanning electron microscope. Percent germination was assessed using the "hanging drop technique" (STANLEY and LINSKENS, 1974). Germination medium was that of Goldy and Lyrene (1983) and consisted of 100 mg/l H_3BO_3, 300 mg/l $Ca(NO_3)_2 \cdot H_2O$, 200 mg/l $MgSO_4 \cdot 7\ H_2O$, 100 mg/l KNO_3, and 10 % sucrose.

Pollen samples for scanning electron microscopy were prepared by dusting pollen onto aluminum stubs covered with double sticky tape. Samples were stored overnight in a vacuum desicator at room temperature and then coated with 40 nm of gold/palladium in a Hammer II (Technics) ion coater at a vacuum pressure of 5×10^{-4} Pa. Pollen grains were examined and photographed at 20 kV and a beam current of 30 pA in a Hitachi S-450 scanning electron microscope (GOLDSTEIN et al., 1981).

Pollen Tube Growth

To study the pathway of pollen tube growth in the style, the technique of Kho and Baer (1968) was used. Four days after pollination (DAP), pistils were collected and fixed in FAA (40% formaldehyde:glacial acetic acid:50% ethanol, 10:5:85, v/v/v) for 24 h. Fixed pistils were rinsed in tap water and softened in a 1 N NaOH solution for 2 h. After washing off the NaOH, pollen tube callose was stained by placing the softened pistils overnight in a 0.1% solution of water soluble aniline blue in 0.1 N K_3PO_4. Pistils were then squashed on a microscope slide in a drop of glycerol and observed with a Leitz

epifluorescent microscope equipped with a 150 W xenon lamp and a BG 38/UG 1 filter combination.

For observation of pollen tubes inside the ovary and for a rapid and direct assessment of pollen tube growth in the style, fresh flowers were collected and hand sectioned at 30-60 μm, since pollen tubes are retained better in thick sections. Staining was again accomplished in a solution of water soluble aniline blue for 1 h. Cross or longitudinal sections of the ovary and pistil were placed in a drop of the staining solution on an uncovered microscope slide, and immediately observed using an epifluorescent microscope.

Fertilization and Early Endosperm and Embryo Development

Ovules were collected and cleared for direct observation under the microscope for assessment of percent fertilization and for the study of early embryo and endosperm development. After pollination, flowers were collected for fixation every 2 days for the first 20 days, and every 2-4 days thereafter. Ovules were dissected out of the ovary and fixed in FPA_{50} (40% formaldehyde:propionic acid:50% ethanol, 5:5:90, v/v/v) for 24 h (HERR, 1974a). Ovules were cleared using Crane's (1978) technique. Fixed ovules were dehydrated in a 50-70-85-100-100% ethanol series for 30 min each. They were then brought to 100% methyl salicylate through 2 changes for 30 min each in a 1:1 and 1:3 ethanol:methyl salicylate mixture. Finally they were transferred to 100% methyl salicylate overnight, and mounted on a Raj slide (HERR, 1974b) in the same clearing solution. Observation of the cleared

ovules was done with a Nikon Normarski differential interference contrast microscope at a magnification of 400x.

For the anatomical studies semi-thin sections (3-4 μm) were made from plastic embedded ovules. Fixation was done in either FAA or in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7) (DAWES, 1971). After fixation, samples were dehydrated in an ethanol series and embedded in JB-4, a glycol methacrylate-based plastic polymer (Polysciences, Inc.). Infiltration of the samples and polymerization of the plastic was done according to the manufacturer's recommendations (Polysciences Data Sheet No. 123, 1982). Sections were cut with a MT 5000 Sorvall Ultra Microtome, using the technique outlined by Bennett et al. (1976). Sections were stained with a 0.05% toluidine blue solution (O'BRIEN and McCULLY, 1981), and permanently mounted in Permount for observation with a Leitz bright field microscope. Pictures were taken using Kodak Panatomic-X film and final magnification of prints were calculated by measuring a standard mm photographed and enlarged in the same way as the prints.

Results

Description of Reproductive Organs

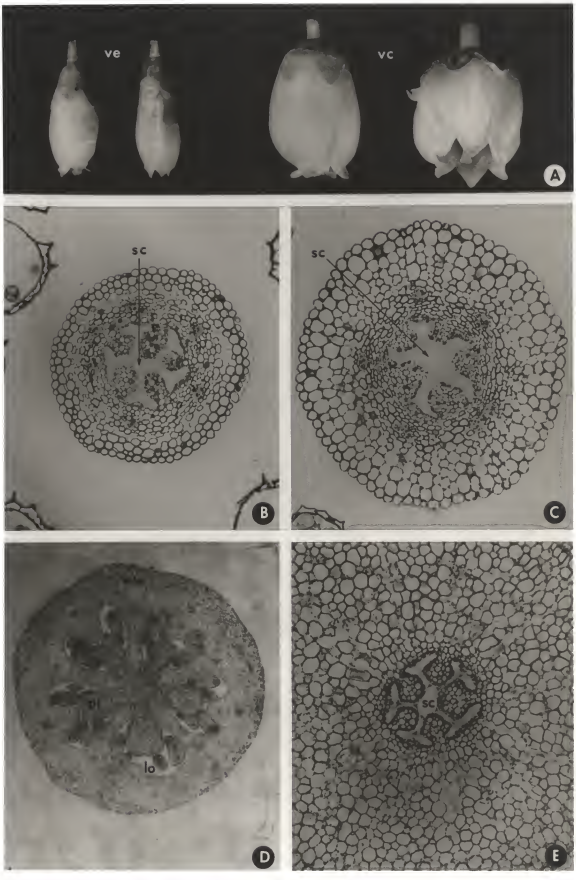
Flowers of both *Vaccinium* spp. under study are produced in racemes. In *V. corymbosum* racemes consist of up to 10 flowers each borne at the end of a 1-2 mm peduncle. The flower peduncle in *V.*

elliottii is up to 5 mm long and there are typically 2-3 flowers per cluster, but up to 6 flowers can be found in some clones.

The general anatomy and morphology of the flowers of both species are similar, except for their size (Figure 1A). Individual flowers possess 5 white or light-pink petals united in a tubular corolla. There are typically 10 stamens and the ovary is inferior (ECK, 1966). Ovaries are commonly divided into 5 locules (Figure 1D), but 4 or 6 are sometimes present. Placentation is axial (BELL and GIFFIN, 1957) and the number of ovules per locule varies both within each locus and from plant to plant. *V. elliottii* possesses 10-20 ovules per locule and *V. corymbosum*, 15-30. Ovules are approximately the same size in both species.

The pistil of these species consists of a filiform style that ends in an unmodified stigma. The style possesses a distinct stylar canal which is typically 5-channelled (Figure 1B,C). This canal is continuous between the stigma and the ovary and, at the time of anthesis, is filled with a mucilaginous secretion that becomes evident at the stigmatic surface. This mucilaginous material is produced by secretory epithelial cells that delimit the interior of the stylar canal throughout its pathway. At the base of the style, the canal becomes very conspicuous with a circular cavity at the center and 5 T-shaped channels departing from it (Figure 1E). Each of the 5 channels communicates with each of the locules through a thin conduit that opens into the locular cavity, which is devoid of the mucilaginous substances. Except for the length of the stylar canal and the amount of parenchymatous tissues surrounding it, there are no major differences in the anatomy of the pistils of these 2 species (Figure 1B,C).

FIGURE 1. A. Typical flowers of *V. elliottii* (ve) and *V. corymbosum* (vc) at anthesis. 3.5x.
B. Cross section of the style of V. elliottii showing a 5-channeled stylar canal. 110x.
C. Cross section of the style of *V. corymbosum* showing a 5-channeled stylar canal. 110x.
D. Cross section of the ovary of *V. elliottii* showing 5 locules with axial placentation. ow: ovary wall; pl: placenta; lo: locule; ov: ovule. 23x.
E. Stylar canal of *V. corymbosum* just before each channel diverts to each of the 5 locules. 110x.

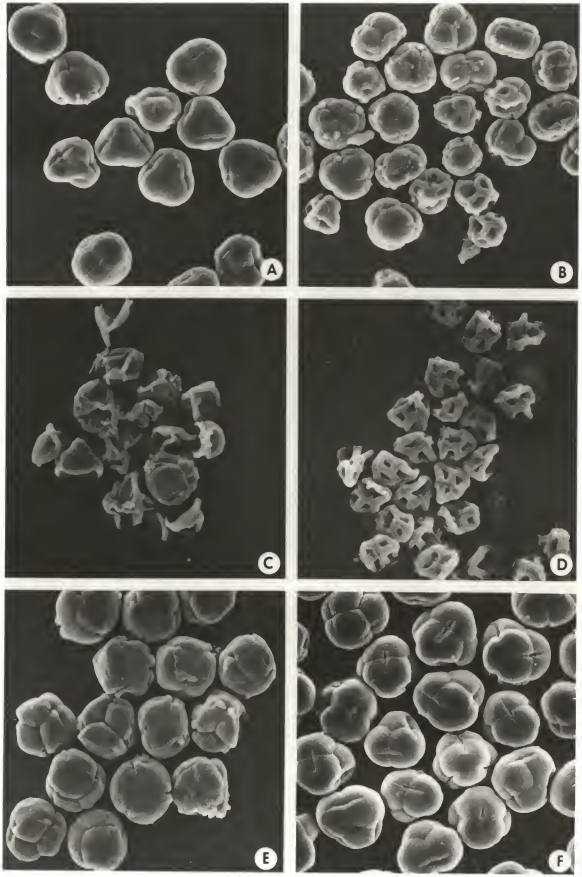

ve
vc
A
sc
B
sc
C
pl
lo
D
sc
E

Vaccinium pollen grains are of the dry type. They are typically shed in tetrahedral tetrads. Individual pollen grains are bicellular, tricolporate with open furrows of medium length, which carry a persistent membrane (operculum) over equatorially elongated pores (LIEUX and GODFREY, 1982). Major morphological differences do not exist between pollen of diploid *V. elliottii* and tetraploid *V. corymbosum*, but the diploid species possesses slightly smaller pollen grains (Figure 2).

Pollen Germination and Tube Growth in Intraspecific Pollinations

Pollen germination usually occurred within the first 24 h in both species. By the end of a 48 h period, most pollen tubes could be observed at the base of the style (Figure 3A), and at 72 h, pollen tubes were growing inside the locules on the surface of the placenta (Figure 3B). Most tubes grew in a straight manner through the stylar canal to the base of the style, filling much of the available space. Two different patterns of callose deposition on the walls of pollen tubes were observed. In some pollinations, pollen tubes showed heavy and almost continuous deposition of callose along the entire length of the tube (Figure 3D). In others, callose deposition occurred mainly at the plugs which are produced at irregular intervals behind the growing tip of the tube (Figure 3C). This variation in the pattern of callose deposition was not related to species or type of cross.

FIGURE 2. Scanning electron micrographs of pollen tetrads of *Vaccinium elliottii* (A-D) and *V. corymbosum* (E,F). 400x. (See text for explanations).


A
B
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D
E
F

Pollen Germination and Tube Growth in Interspecific Pollinations

The timing of pollen tube growth in interspecific pollinations followed almost the same pattern as in intraspecific pollinations. In general, 48 h after pollination, pollen tubes could be seen at the base of the style, but in smaller numbers than in intraspecific pollinations.

Pollen germination and tube growth was always better when *V. corymbosum* was used as male parent. Large variation in pollen germination and tube growth was observed when *V. elliottii* was the male parent (Table 1).

TABLE 1. Pollen germination of some *Vaccinium corymbosum* and *V. elliottii* cultivars and selections used in the crossing experiments.

Cultivar or selection	% Pollen germination[z]	Cultivar or selection	% Pollen germination[z]
V. corymbosum		*V. elliottii*	
Avonblue	68	FL 82-148	30
Flordablue	42	FL 80-44	6
Sharpblue	50	FL 81-196	10
NC-1688	95	FL 80-67	2
FL 79-25	30	FL 82-226a[y]	80
FL 64-65	90	FL 82-226b[y]	8
FL 64-76	85	FL 82-226c[y]	0
Mean[x]	60+24		19+29

[z]% germination was estimated from a mixture of pollen from 5 flowers from each clone. More than 1000 pollen grains were counted.

[y]a, b, and c are 3 different plants represented in the 82-226 pollen mixture.

[x]mean + standard deviation.

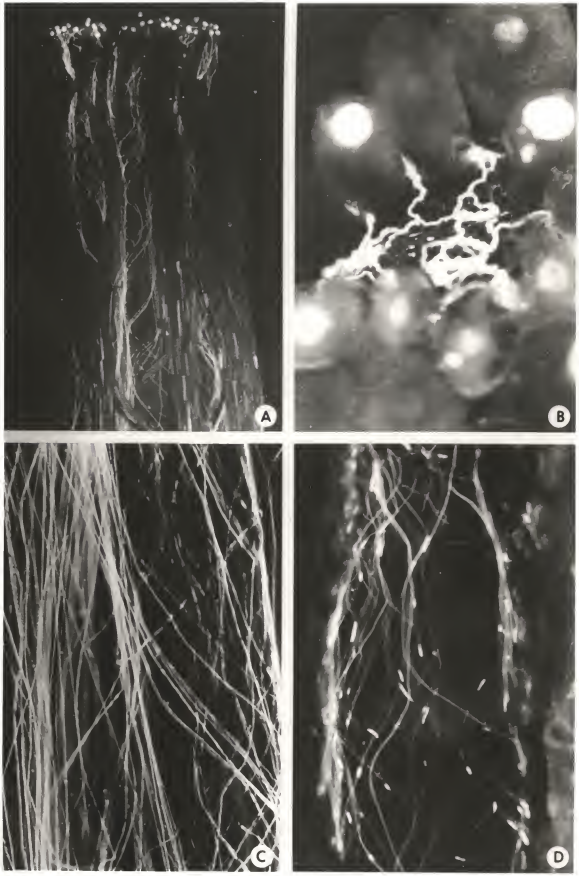
FIGURE 3. Pollen tube growth in intraspecific pollinations of *Vaccinium corymbosum* and *V. elliottii*.

A. Pollen tubes growing down the style in *V. elliottii*. 36x.

B. Pollen tubes growing on the surface of the placenta of *V. corymbosum*. 140x.

C. Continuous deposition of callose in pollen tube walls. 140x.

D. Deposition of callose mainly at the plugs formed behind the growing tips of the pollen tubes. 140x.


A
B
C
D

This variability in pollen germination may have had 2 causes. First was the fact that pollen stored for up to 90 days under refrigeration was often used when *V. elliottii* was the male parent, and it was observed that germination of stored pollen was usually lower than that of recently collected pollen. Storage seemed less detrimental to pollen viability in *V. corymbosum*, although long storage slightly decreased percent pollen germination in this species. Secondly, it was found that *V. elliottii* had a high degree of pollen sterility in natural stands. *In vitro* assessment of pollen viability in these two species showed that *V. corymbosum* ranked consistently higher than *V. elliottii* in percent pollen germination (Table 1).

Observation of pollen grains with scanning electron microscopy showed that inviability of pollen in *V. elliottii* was due to a developmental failure leading to the formation of unfilled pollen grains, irregularly developed tetrads, and grains with abnormal exine morphology, rather than to failures in the germination process. Figure 2 A-D shows pollen of 4 different selections of *V. elliottii* with increasing degrees of sterility. Pollen of 2 *V. corymbosum* selections is shown for comparison (Figure 2E,F). This abnormal pollen of *V. elliottii* was shed in clusters, apparently due to the presence of a sticky substance on the surface of the grains. Normal pollen, on the other hand, was shed in single tetrads that did not stick to each other.

Another abnormality in the behavior of pollen tubes in interspecific crosses was observed at the ovary locules. Although pollen tubes were observed at the base of the style in interspecific crosses, they were not seen growing into the locular cavities or on the surface of the placenta in the same numbers as in intraspecific crosses

(Figure 3B). This was particularly true when *V. elliottii* was used as male parent. Assessment of the percent of fertilized ovules 8 DAP confirmed this observation. Table 2 shows that in some cases pollen tubes were observed at the base of the style, but no fertilized ovules were detected.

Fertilization and Development of Embryo and Endosperm in Intraspecific Pollinations

Seed development in *V. corymbosum* has been described in detail (EATON and JAMONT,1966; STUSHNOFF and HOUGH, 1969), however, nothing has been reported on seed development in *V. elliottii*. Our observations indicate that seed development in *V. elliottii* is not substantially different from that of *V. corymbosum* or from that of most other *Vaccinium* species (BELL, 1957; STUSHNOFF and PALSER, 1969).

As in most *Vaccinium* spp., ovules of *V. elliottii* are anatropous with a single integument that elongates over the nucellus forming a long micropyle. In *Vaccinium,* the nucellus is ephemeral and disappears early during megagametophyte development (STUSHNOFF and PALSER, 1969), so the embryo sac is two-thirds surrounded by a well differentiated endothelium or integumentary tapetum, which will later enclose the endosperm proper. The micropylar end of the embryo sac is not surrounded by the endothelium and will later differentiate into the micropylar haustorium.

Embryo sac development is of the *Polygonum* type (MAHESHWARI, 1950); with 3 elongated antipodals, one of which is particularly long; 2 small densely cytoplasmic synergids located at each side of a

conspicuous egg cell; and 2 polar nuclei usually found adjacent to the egg cell (Figure 4A).

Fertilization itself was difficult to observe, but pre- and postfertilization events followed similar patterns in both species. Although pollen tubes were observed at the base of the style 2 DAP, fertilization did not occur until 5-7 DAP. It was not possible to determine whether penetration of the ovules by the pollen tubes was delayed or whether discharge of the sperm cells and subsequent fusion with the egg and polar nuclei took longer, but pollen tubes were seen penetrating the micropyle as early as 4 DAP. Actual fusion of the gametes was never observed, so the first evidence that fertilization had occurred was the observation of the first primary endosperm nucleus division, which normally occurred about 6 DAP (Figure 4B).

As soon as the primary endosperm nucleus divided, a series of morphological changes occurred in the flowers, including rapid swelling of the ovary walls and abscission of the pistil. In flowers where fertilization did not take place, the pistils did not abscise but remained attached to the flower until the pedicel abscised. Pistil abscission following pollination was a good indication that fertilization had occurred.

The number of fertilized ovules within each locule and within each fruit varied considerably, but it appeared that a few fertilized ovules were sufficient to sustain fruit development in both species.

Embryo and endosperm development in *V. elliottii* followed a pattern similar to that of *V. corymbosum*, but endosperm development

FIGURE 4. Embryo and endosperm development in *V. elliottii* after intraspecific crosses.

A. Embryo sac at anthesis. Note the 2 densely cytoplasmic synergids (sy), the egg cell (eg), the 2 polar nuclei (pn), and the antipodals (an). 212x.

B. Embryo sac 6 DAP, showing a 4-celled endosperm (ep). 212x.

C. Endosperm (ep) development 10 DAP, showing the invasion of the micropylar haustorium (mh) by endosperm cells. 106x.

D. Endosperm (ep) and undivided zygote (zy) 12 DAP. 106x.

E. First zygotic division (zy) 18 DAP. Note the densely cytoplasmic micropylar haustorium (mh) and that the zygote is well passed the constriction (ct) into the enposperm proper (ep). 106x.

F. Linear pro-embryo 20 DAP. 106x.

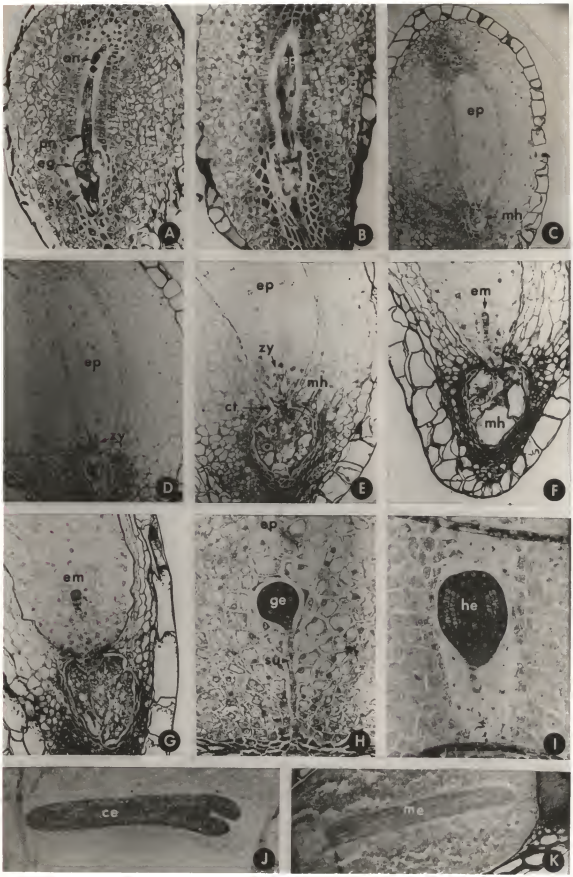
G. First division of the terminal cell of the linear pro-embryo 22 DAP. 106x.

H. Globular pro-embryo (ge) at the end of a long suspensor (su) surrounded by endosperm cells (ep), 26 DAP. 212x.

I. Heart-shaped pro-embryo (he) 33 DAP. 212x.

J. Cotyledonary embryo (ce) 38 DAP. 106x.

K. Mature embryo (me) 43 DAP. 53x.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usually started somewhat earlier in *V. elliottii*. Following fertilization, the egg cell shrank and became densely cytoplasmic. As the endosperm started dividing and its cells invaded the micropylar haustorium, the zygote was displaced toward the chalazal end of the ovule and became very difficult to distinguish from endosperm cells, as these too became fairly dense towards the micropylar end (Figure 4C,D). Once the zygote passed the constriction that separates the micropylar haustorium from the endosperm proper, it became clearly distinguishable because of its small size and dense cytoplasm (Figure 4D,E).

About 2 weeks after pollination, when the endosperm proper was over 130 cells in size, the zygote started to divide. About 20 DAP it had approximately 10 cells, most of which later constituted the suspensor (Figure 4F). From this point on, embryo development was rapid, going successively through the globular (Figure 4H), heart shape (Figure 4I), torpedo, and cotyledonary stage (Figure 4J) in less than 7 days. Forty DAP, the embryo was almost fully developed (Figure 4K) and occupied about 30 % of the seed cavity. In other *Vaccinium* spp. a mature embryo occupies up to 80 % of the seed cavity (EDWARDS et al.,1972).

Between 16 and 22 DAP, endosperm cells started showing heavy depositions of starch granules. The starch granules increased in size as the endosperm continued its development (Figure 4H,I). This starch deposition caused the endosperm to change abruptly from an almost translucent structure to a white mass of cells that were clearly distinguishable as the ovules were dissected.

Fertilization and Development of Embryo and Endosperm in Interspecific Croses

Despite the difference in pistil length in the 2 species, the timing of fertilization in interspecific crosses appeared almost identical to that of intraspecific pollinations, irrespective of which species was used as female parent. Thus, fertilization occurred about 5 DAP, and 1 day later, the endosperm had 2-4 cells.

Estimation of the number of fertilized, unfertilized, and non-fertile ovules was made by observing embryo sac development 8 DAP using cleared ovules and interference contrast microscopy (Table 2). Fertilized ovules were considered to be those which showed division of the primary endosperm nucleus, which 8 DAP usually showed 8-16 cells (Figure 5A). The technique did not allow, however, for an unequivocal assessment of the stage of zygote development. Despite this limitation, the technique was considered adequate to roughly estimate the percent of fertilized ovules, since single fertilization, i.e. fertilization of either the polar nuclei or the egg cell alone, is very rare (MAHESHWARI, 1950). Non-fertile ovules were easily distinguishable because they usually remained the size they were at the time of pollination and showed no differentiation of the embryo sac (Figure 5B). Unfertilized ovules, on the contrary, grew almost to the same size as those that were fertilized, the embryo sac was fully differentiated, and both the egg cell nucleus and the polar nuclei were abnormally large inside highly vacuolated cells (Figure 5C). Also, the polar nuclei moved close to the egg cell nucleus and in many instances they moved inside the cavity that later hosted the micropylar haustorium (Figure 5D).

FIGURE 5. Early embryo and endosperm development as seen with Normarski differential interference contrast microscopy.
A. Eight-celled endosperm (ep) 8 DAP. 570x.
B. Non-fertile ovule with completely undifferentiated embryo sac. (440x).
C, D. Unfertilized ovules. Note the egg cells with a prominent nucleus (eg), the two polar nuclei (pn), and a well differentiated endothelium (en). 570x.

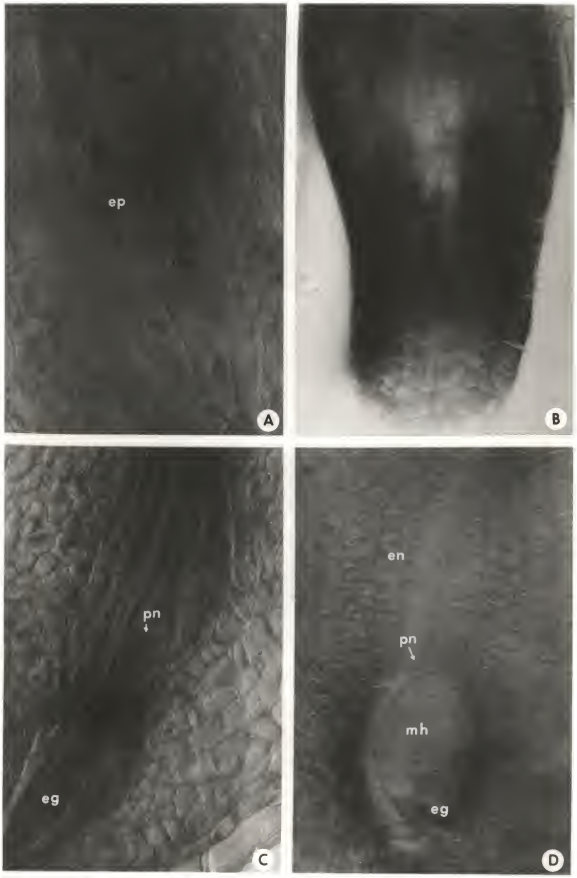
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TABLE 2. Pollen tube growth and percent fertilized, unfertilized, and non-fertile ovules in various crosses between *Vaccinium corymbosum* and *V. elliottii*[z].

Cross	Pollen tube growth[y]	Fertilized %	Unfertilized %	Non-fertile %
V. corymbosum (female) x *V. elliottii* (male)				
Sharpblue x FL 81-198[x]	+++	42.1	46.9	11.0
Avonblue x FL 82-226[w]	+	0.0	72.0	28.0
Flordablue x FL 82-227[w]	++	0.0	73.9	26.1
FL 64-65 x FL 82-226	+	0.0	75.0	24.7
FL 64-76 x FL 82-227	++	0.0	87.2	12.8
FL 79-25 x FL 80-67	++	1.2	82.1	16.7
FL 79-26 x FL 82-227	+	1.2	87.8	11.0
NC-1688 x FL 81-196	+	9.8	67.9	22.3
Mean		6.8	74.1	18.2
V. elliottii (female) x *V. corymbosum* (male)				
FL 81-196 x Sharpblue	+++	63.7	22.6	13.7
FL 82-148 x NC-1688	+++	55.8	30.2	14.0
FL 80-67 x NC-1688	+++	30.8	35.9	33.3
FL 80-44 x NC-1688	+++	27.6	36.2	36.2
Mean		44.5	31.2	24.3

[z]Values are the average of a sample of 10 flowers collected 8 day after pollination.

[y]+ 1 to 10, ++ 10 to 30, +++ more than 30 pollen tubes observed at the base of the style.

[x]Pollinations made with fresh pollen.

[w]82-226 and 82-227 are two different pollen mixtures collected from randomly selected wild plants.

Percent female sterility, as assessed by this technique, varied according to clone and species, with *V. elliottii* showing slightly higher female sterility than *V. corymbosum* (Table 2).

As was noted for intraspecific pollinations, endosperm

development in *V. elliottii* started a little earlier than in *V. corymbosum*, and continued its development at the same rate as in intraspecific pollinations, up to 10-12 DAP. At this time it consisted of about 16 cells (Figure 6A-D). From then on, the endosperm stopped dividing, cells acquired irregular shapes, and in some cases karyokinesis was not followed by cytokinesis, so a few cells of the endosperm appeared polynucleated (Figure 6E). Meanwhile the zygote was still undivided and being displaced out of the micropylar haustorium (Figure 6F). By 20 DAP, most of the endosperm cells had collapsed, and very few nuclei could be distinguished (Figure 6H). Since the integument had continued to grow, 20 DAP the remnants of the endosperm cells were crushed in the middle of the central cavity of the ovule (Figure 6I), and the endothelium had disintegrated.

As the endosperm proper degenerated, cells in both the micropylar and chalazal haustoria also stopped dividing and their vacuoles started to grow (Figure 6G). In contrast, haustorial cells of ovules from intraspecific crosses continued to divide vigorously and appeared highly cytoplasmic (Figure 4D-G).

Although in the vast majority of the cases deterioration of the endosperm occurred before the first division of the zygote took place, there were a few fruit on some plants that carried ovules in which deterioration of the endosperm did not occur, and embryo and endosperm development continued in a way similar to that of intraspecific crosses. This condition was particularly evident in those genotypes that to some extent possessed the ability to develop

FIGURE 6. Embryo and endosperm development in *V. elliottii* after interspecific crosses.

A. Unfertilized embryo sac 4 DAP showing the egg cell nucleus (eg) and the 2 polar nuclei (pn). 260x.

B. First transverse division of the primary endosperm nucleus (pe) 8 DAP. 130x.

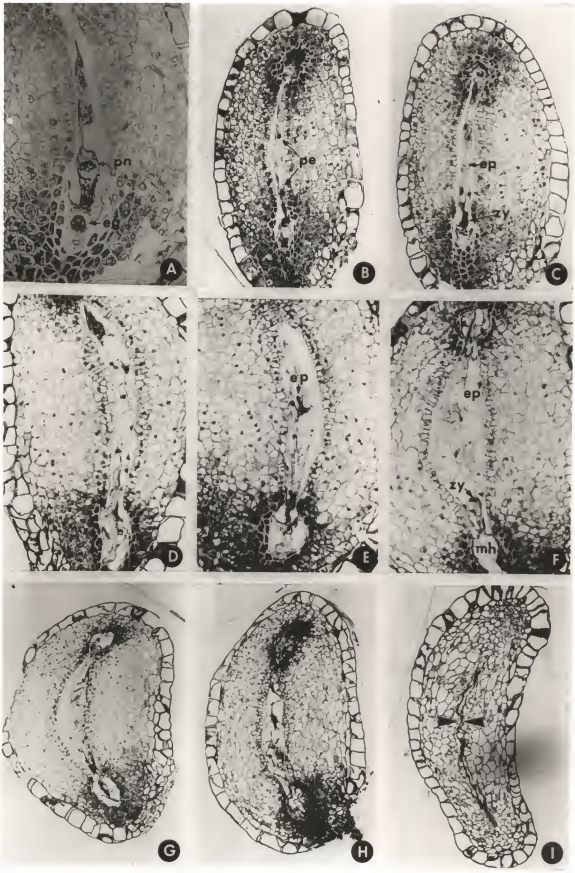
C. Second longitudinal division of the endosperm (ep) 8 DAP. Note the densely cytoplasmic undivided zygote (zy). 130x.

D, E. Endosperm (ep) development 10 DAP showing an almost normal developing pattern (D) and an abnormal one (E) where cell division is irregular and nuclei form clusters (ep). 130x.

F. Endosperm and zygote development 12 DAP. Note the irregular shapes of endosperm cells (ep) and the undivided zygote which is already out of an empty micropylar haustorium (mh). 130x.

G, H. Different stages of endosperm degeneration 14 (G) and 16 (H) DAP. 65x.

I. Remnants of endosperm cells crushed at the center of the ovule (arrows) 18 DAP. 65x.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parthenocarpic fruit (e.g. FL 81-196). In cases where the endosperm stopped developing early on during seed formation, the fruit abscised soon after the first evidence of endosperm deterioration was detected. In others, the fruit continued its development for up to 30 DAP without abscising, but examination of these fruits at this stage of development only rarely showed ovules with some indication of embryo or endosperm development.

When *V. corymbosum* was used as a female parent, most ovules showed endosperms that did not develop beyond the 4 celled stage (Figure 7A-C). From then on, deterioration followed an almost identical pattern as in *V. elliottii* (Figure 7D-E), but more embryos continued their development and some eventually reached maturity. Endosperm degeneration occurred at various stages of development, but usually before the cotyledonary stage was attained. Figure 7G,H shows an example of endosperm degeneration 24 DAP. In this case, cells show progressive vacuolation toward the chalazal end and starch deposition has occurred only at the micropylar end. Another example (Figure 7F) shows an endosperm 21 DAP where cells surrounding the embryo near the constriction have already collaspsed. In both these cases the embryo was not found, so it had already aborted or was still undivided and could not be distinguished. By 21 DAP non-hybrid embryos in *V. corymbosum* had passed the globular stage and were easily distinguishable. This is an indication that, at least in some cases, the zygote did not divide despite an almost normal endosperm development.

Hybrid embryos that were able to develop beyond the cotyledonary stage usually reached maturity, but developed more slowly

FIGURE 7. Embryo and endosperm development in *Vaccinium corymbosum* after interspecific crosses.

A. Unfertilized embryo sac 4 DAP, showing the egg cell (ec) and polar nuclei (pn). 260x.

B. First division of the primary endosperm nucleus (pe) 6 DAP. Note a well differentiated endothelium (en). 260x

C. Second division of the primary endospern nucleus 8 DAP. The zygote (zy) is almost the same size as before fertilization. Endosperm cells are densely cytoplasmic and show little growth after division. 260x.

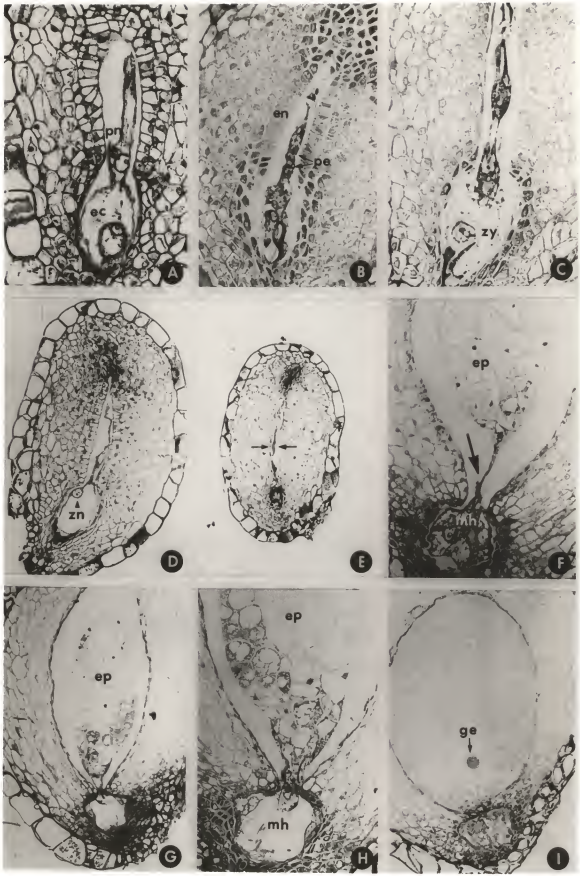
D. Endosperm cells had collapsed 12 DAP and only the zygote nucleus (zn) remains. 130x.

E. Remnants of endosperm cells being crushed at the center of the ovule (arrows) 18 DAP. 65x.

F. Cells of the endosperm proper (ep) collapsed at the constriction (arrow) 21 DAP. Note the vacuolated micropylar haustorium (mh). 130x.

G, H. Endosperm cells (ep) showing partial starch deposition and large vacuoles 24 DAP. Note that the micropylar haustorium (mh) is empty. G,65x; H,130x.

I. Globular embryo (ge) 30 DAP. 65x.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and attained a smaller size than non-hybrid ones. Figure 7I shows an example where a hybrid embryo is just at the globular stage of development 30 DAP. A non-hybrid embryo would have passed the cotyledonary stage of development 30 DAP (STUSHNOFF and PALSER, 1969). More ovules reached maturity when *V. corymbosum* was the female parent.

These differences in the pattern of abortion can also be observed by looking at ovule growth in both intra- and interspecific crosses. Figure 8A,B shows typical ovule growth curves that were constructed using the size of the largest ovules in randomly selected flowers at various dates after pollination and fitted to the Gompertz function (CAUSTON, 1969). It can be observed that when *V. elliottii* was the female parent, the first change in the rate of ovule growth occurred 12 DAP and that ovules from interspecific crosses attained their maximum size 18 DAP with little variation in size among sample dates from then on. When *V. corymbosum* was used as female parent, the rate of ovule growth changed somewhat earlier (10 DAP) and there was much variation in the maximum ovule size attained at later dates, showing that abortion in this case occurred over a longer period of time.

Discussion

Various abnormalities which lead to embryo abortion were detected throughout pollination and embryo and endosperm development in crosses between *V. corymbosum* and *V. elliottii*. Although this strong cross-incompatibility was observed no matter which species was used as

FIGURE 8. Ovule development after intraspecific (e x e, c x c) and interspecific (e x c, c x e) reciprocal crosses between *Vaccinium corymbosum* and *V. elliottii*. Regression lines fitted to the Gompertz function according to the following equations:

e x e: Y=2.05*exp(-2.47*exp(-0.11*X))
e x c: Y=1.21*exp(-1.47*exp(-0.15*X))
c x c: Y=1.97*exp(-3.64*exp(-0.13*X))
c x e: Y=0.95*exp(-0.99*exp(-0.10*X))

The parameters for these equations were estimated using the NLIN procedure of the Statistical Analysis System (SAS).

female parent, there were different sites and various degrees of expression of the incompatibility barriers depending on the direction of the cross and the clones involved.

It was found that abnormalities occurring before fertilization were only important when *V. elliottii* was the male parent. The low pollen fertility found in many unselected plants of this species was surprising, because it is a diploid and should not show the sterility problems that result in some polyploids from multivalent paring of homologous or homoeologous chromosomes (SANFORD, 1983). Low pollen fertility has also been reported in *V. darrowi* (GOLDY and LYRENE, 1983), another diploid species, and male sterile individuals have been found in *V. angustifolium*, a tetraploid species (AALDERS and HALL, 1963).

Pollen sterility was correlated with egg sterility (r=0.72) in *V. elliottii*, suggesting that infertility in this species might be of cytogenetic origin. These observations suggest that careful selection for high fertility should be made if this species is to be used in breeding, particularly because there is large variability for this trait. Another fertilization abnormality found when *V. elliottii* was the male parent was the failure of pollen tubes to penetrate the ovarian cavity of *V. corymbosum* after the tubes had reached the base of the style. This same situation has been reported in interspecific crosses in *Rhododendron* (KHO and BAER, 1970; WILLIAMS et al.,1982) and *Panicum* (BURSON and YUNG, 1983). In the experiments reported here, failure of pollen tube penetration into the ovary was observed only when the short-styled species was used as the male parent, suggesting that differences in the length of the styles and pollen tubes were

responsible for this abnormality, as has been reported in other species (KHO and BAER, 1973). This seems unlikely, however, because the phenomenon was inconsistent and several cases were observed where fertilization took place in spite of the differences in style length between these 2 species.

Another general observation was that even in those interspecific crosses where fertilization occurred normally, there was a strong postfertilization barrier that induced embryo abortion very early after fertilization, usually before the zygote started dividing. Similar results have been reported by Aalders and Hall (1961), who found that in crosses between diploid *V. myrtilloides* and tetraploid *V. angustifolium* the endosperm formed only 6-8 cells before it started to disintegrate.

This indicates that the triploid block is very strong in blueberries, but gives little information about its causes. It has been hypothesized that embryo abortion in heteroploid crosses results from a genetic imbalance between maternal and paternal genomes in the endosperm (KIHARA and NISHIYAMA, 1932; NISHIYAMA and INOMATA, 1966; NISHIYAMA and YABUNO, 1978; JOHNSTON et al.,1980). This imbalance impedes normal endosperm development and consequently induces embryo abortion. Whether embryo abortion resulted from endosperm breakdown remains uncertain in blueberry. It was observed, particularly when *V. corymbosum* was the female parent, that in some cases the endosperm developed in an almost normal way for up to 21 DAP while the zygote remained undivided in an apparent rest stage. Normally, by 21 DAP an embryo will be past the heart shape stage of development. This is an indication that, at least in some cases, endosperm breakdown is not the

direct cause of zygote malfunction. Similar observations have been made with heteroploid crosses in *Citrus* (ESEN and SOOST, 1973), *Lycopersicon* (COOPER and BRINK, 1945) and *Trifolium* (KAZIMIERSKA, 1980). In somatic cell fusion experiments with *Nicotiana*, no hybrids have been produced in heteroploid crosses where hybrids can not be obtained using sexual crossing procedures. This is due to either the inability of the heterokaryon to divide or to the partial or total elimination of the chromosomes of one of the species following fusion (HARMS, 1983).

The slight differences in pattern of embryo and endosperm development found with reciprocal crosses are of little consequence if one is trying to obtain interspecific hybrids. Nevertheless, the facts that when *V. corymbosum* was used as female more ovules showed embryo development, and that there was variability for the stage of development at which abortion occurred may have some implications when designing crossing strategies.

SECTION IV

IN VITRO ATTEMPTS TO OVERCOME THE CROSS-INCOMPATIBILITY BETWEEN *Vaccinium corymbosum* L. AND *V. elliottii* CHAPM.

Introduction

Embryo culture has been used in a number of species in efforts to overcome postfertilization barriers which prevent the recovery of hybrids from interspecific crosses (RAGHAVAN, 1978). Experiments with various species have demonstrated that the earlier abortion takes place, the harder it is to rescue the hybrid embryo (RAGHAVAN, 1976, 1977, 1980; MONNIER, 1978).

It has been found in crosses between *Vaccinium corymbosum* and *V. elliotti* that embryo abortion usually occurs before the zygote starts dividing (see Results, Section III). This represents a problem because is difficult to remove and culture the single-celled zygote from the embryo sac. However, methods have been developed that enable the culture of hybrid embryos without dissecting them from maternal tissues. Both *in ovary* (INOMATA, 1968; WATANABE, 1977; MATSUZAWA, 1978; TAKESHITA et al.,1980) and *in ovulo* (WAKISUKA and NIKAJIMA, 1974; REED and COLLINS, 1978; STEWART and HSU, 1978; TAKESHITA et al.,1980) embryo culture have been successfully used to rescue hybrid embryos from interspecific crosses.

A series of experiments was conducted to evaluate these 2 embryo rescue techniques in reciprocal crosses between *V. corymbosum* and

V. elliottii. Several factors were studied with respect to *in ovulo* embryo rescue. These included the effect of the developmental stage of embryo when ovules were placed in culture, the effects of adding various components to the nutrient medium, the influence of maternal tissues when culturing the ovules, and the effect of auxins applied to the ovaries previous to culture of ovules.

Factors studied for *in ovary* embryo culture included the effect of the stage of embryo development at the time ovaries were placed in culture, the effects of several nutrient medium components, and the effect of the position of the ovary in the culture medium. In addition, some experiments were conducted in an effort to increase the frequency of fertilized ovules using *in vitro* pollination.

Materials and Methods

Plant materials and crossing procedures were the same as indicated in the previous section (see Materials and Methods, Section III). Experiments with *in ovulo* embryo culture were done mainly during the 1982 flowering season. Ovules were extracted from flowers collected from greenhouse-grown plants at various dates after pollination. After collection, flowers were immediately surface sterilized by immersing them for 15 min in 100 ml of a 1% sodium hypochlorite solution (ca. 20% Clorox) to which 1 drop of polyoxyethylene sorbitan monolaurate (Tween 20) had been added. They were then rinsed 5 times in sterile, distilled water. Ovules were dissected out of the ovary under a laminar flow hood with the aid of a dissecting microscope and the

appropiate dissecting tools. Ovules were more easily removed undamaged if the walls of the ovaries were removed first (Figure 9A), and the ovules inside each locule were removed attached to the placenta (Figure 9B).

Extracted ovules were then plated on small Petri dishes containing 5 ml of a highly modified Murashige and Skoog (1962) medium (Table 3). All experiments were conducted using this same basic culture medium.

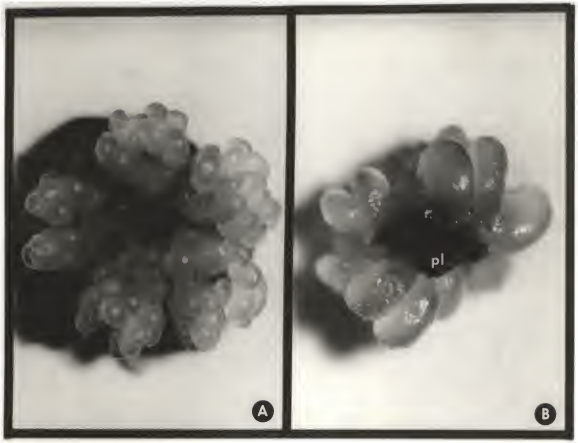
To study the effect of time of excision, ovules from both intra- and interspecific crosses were extracted from each of the 5 locules and placed in culture attached or detached from placental tissues. Ovules were placed in culture every 7 days, starting about 15 DAP and for a period of 4 weeks. Cultures were kept in the dark, at 25°C for about 90 days. Ovules that developed into seed were then transfered to 0.6% water-agar and placed under indirect sunlight to induce germination. Red light has been demonstrated to be essential for germination of blueberry seed (GALLETTA, 1975).

The effect of auxins in delaying embryo abortion was studied following the application to the external part of the ovary of 1% naphthalene acetamide in lanolin paste immediately after pollination (DARROW, 1956). Following this treatment ovules were extracted and cultured 20 and 27 DAP as described above.

Various nutrient media components were evaluated after their addition to the basic culture medium. Coconut milk (5%), 5 different sucrose levels (15-20-30-40-60 mg/l), and 3 different vitamin levels (1x-2x-3x normal concentration of myo-inositol, nicotinic acid,

FIGURE 9. Ovules as they were planted in the nutrient medium.
A. Flower with the ovary walls removed.
B. Ovules from one of the locules as they were plated attached to the placental tissues.

FIGURE 10. Method used to plate the cultured ovaries.
A. Two thirds of the whole young fruit were inserted into the solid nutrient medium.
B. The basal 1/3 of the fruit was cut, and the remainder of the fruit was inserted 1/2 way into the nutrient medium, with its calix upwards.
C. The fruit was cut longitudinally, and both halves were placed with the cut surface in direct contact with the nutrient medium.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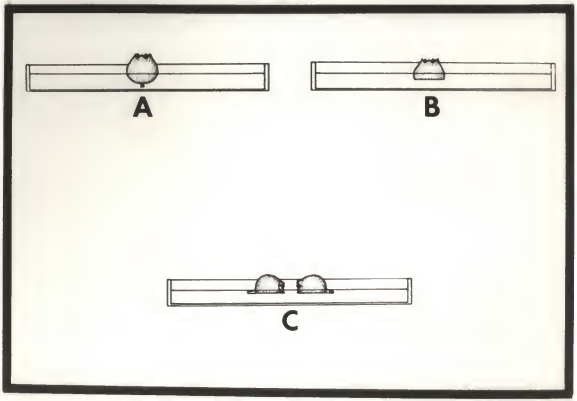
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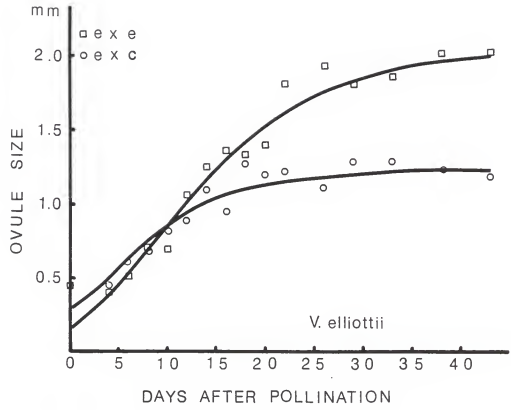
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OVULE SIZE
V. elliottii
0 5 10 15 20 25 30 35 40
DAYS AFTER POLLINATION

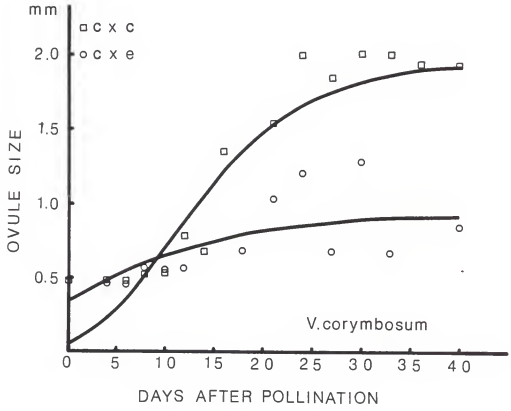

mm
□ c x c
○ c x e
2.0
1.5
1.0
0.5
OVULE SIZE
V. corymbosum
0 5 10 15 20 25 30 35 40
DAYS AFTER POLLINATION

TABLE 3. Modified Murashige and Skoog medium used for *in ovulo* and *in ovary* embryo culture of *Vaccinium corymbosum* and *V. elliottii*[z].

Constituent	Concentration (mg/l)
$Ca(NO_3)_2 \cdot 4 H_2O$	684.0
NH_4NO_3	400.0
KNO_3	190.0
$MgSO_4 \cdot 7 H_2O$	370.0
KH_2PO_4	370.0
Na_2EDTA	74.4
$FeSO_4 \cdot 7 H_2O$	55.8
$MnSO_4 \cdot H_2O$	22.3
$ZnSO_4 \cdot 7 H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2 H_2O$	0.25
$CuSO_4 \cdot 5 H_2O$	0.025
$CoCl_2 \cdot 6 H_2O$	0.025
Myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxin . HCl	0.5
Thiamine . HCl	0.1
Glycine	2.0
Casein hydrolysate	500.0
Sucrose	30,000.0
Agar	4,000.0

[z]pH adjusted to 5.7 with HCl before autoclaving at 103 kPa (121°C) for 20 min.

pyridoxin, and thiamine) were tested. For the evaluation of various growth regulators, a factorial experiment was conducted in which 3 levels (0-2-4 mg/l) of 2-isopentyl adenine (2iP) and 3 levels

(0.0-0.5-1.0 mg/l) of 2,4 dichlorophenoxyacetic acid (2,4-D) were tested with or without adding 0.2 mg/l of succinic acid-2,2-dimethylhydrazide (Alar). Further testing of growth regulators included addition of 0.006 mg/l of 4-amino-3,5,6-trichloropicolinic acid (Picloram), 0.25 mg/l of ABA, and 1 mg/l of the antiauxin 7-azaindole.

Experiments with *in ovary* embryo culture were done mainly during the 1983 flowering season, using the same general procedures used for *in ovulo* embryo culture. In this case the following media components were tested: sucrose (15-30-60-120 g/l), alanine (0.00-0.09-0.45-0.89 g/l), glutamine (0.00-0.15-0.75-1.45 g/l), proline (0.58 g/l), and a combination of alanine, glutamine, and proline at 0.45, 0.73, and 0.58 g/l respectively. Growth regulators tested included picloram (0.006 mg/l) and 2iP (5 mg/l). All ovaries were placed in culture between 11 and 24 DAP.

To study the effect of the position of the ovaries in the culture medium, the following treatments were tested: a) two thirds of the whole young fruit was inserted into the solid nutrient medium (Figure 10A); b) the basal 1/3 of the fruit was cut, and the remainder of the fruit was inserted 1/2 way into the nurient medium, with its calyx upwards (Figure 10B); and, c) the fruit was cut longitudinally, and both halves were placed with the cut surface in direct contact with the nutrient medium (Figure 10C).

Several *in vitro* pollination experiments were conducted using basically the same general procedures and plant material descibed for the culture of ovules and ovaries. For pollination of naked ovules, pollen was directly sprinkled onto ovules attached to placental tissues that had been placed in culture at anthesis. The culture medium

consisted of either the basic medium used for *in ovulo* and *in ovary* embryo culture (Table 3) or of the pollen germination medium used for assessing pollen viability (see Materials and Methods, Section III) with or without solidification with 0.4% agar. Pollen used for pollination was obtained from flowers that were collected just previous to anthesis, surface sterilized, and allowed to open under asceptic conditions. Pollinated ovules were kept in the dark at 25°C.

For *in vitro* pollination of whole flowers, flowers were collected just before anthesis, surface sterilized, emasculated, and pollinated under a laminar flow hood before planting them upwards in vials containing 10 ml of nutrient medium (Table 3). In all cases flowers were planted with the basal portion of the ovary removed (Figure 10B). Pollinated flowers were placed under a light bench at 25°C for a period of 30 days. Pollen tube growth in the style and ovary was observed, following staining with aniline blue, with an epifluorescent microscope (see Materials and Methods, Section III).

Results and Discussion

In ovulo Embryo Culture

A series of preliminary experiments demonstrated that ovules cultured detached from the placenta turned brown and died during the first 10 days in culture. This observation is in agreement with other studies which have also demonstrated that ovules grow best when they are cultured attached to placental tissues (RAGHAVAN, 1976).

A physiological role for placental tissues in ovule development has been proposed for some species (RANGAN,1982). In blueberry this response is more likely the result of less damage to the ovules when they are dissected attached to the placenta, since occasionally some ovules will grow even if they are not attached to maternal tissues. Since the number of detached ovules that did not die shortly after plating was very small, subsequent experiments considered only the culture of ovules attached to maternal tissues.

Culture of ovules attached to placental tissues resulted in various degrees of success depending on the stage of embryo development at the time ovules were placed in culture. Table 4 shows the effect of time of excision on seed development after intraspecific crosses. As many as 8.9% of the ovules of *V. elliottii* developed in culture into mature seeds, even if they were excised as early as 15 DAP, at a time when the zygote had just started to divide (see Results, Section III). As the embryo advanced in maturity, there was an increase in the percent of ovules capable of developing in culture up to 29 DAP, when embryos had reached the cotyledonary stage of development. From then on, there was no further increase in the percent of seed that attained maturity in culture.

A similar trend was observed when ovules of *V. corymbosum* were placed in culture, however, a lower percent of ovules completed their development when they were cultured at proembryonic stages of development, i.e. before 29 DAP. As in the case of *V. elliottii*, once the cotyledonary stage of embryo development was attained, there was no further increase in the percent of ovules capable of developing in culture.

TABLE 4. *In ovulo* culture of embryos from intraspecific crosses in *Vaccinium* as affected by the stage of embryo development at the time of excision.

Days after pollination	Ovules placed in culture	Ovules that developed into seeds		Seed germination	
V. elliottii	No.	No.	%	No.	%
15	225	20	8.9	1	5.0
22	150	29	19.3	2	6.9
29	150	39	26.0	3	7.7
36	225	143	63.5	7	4.9
43	225	131	58.2	13	9.9
50	150	96	64.0	7	7.3
V. corymbosum					
14	250	6	2.4	0	0.0
21	250	3	1.2	0	0.0
28	375	35	9.3	11	31.4
35	125	97	77.6	43	44.3
42	125	61	48.8	21	34.4
49	125	69	55.2	33	47.8

Germination of the cultured seed was low, particularly in *V. elliottii*. This may be explained, at least in part, by the fact that warm-season germination of blueberry seed is normally difficult.

Table 5 shows similar data, but for interspecific crosses. Less than 2% of the cultured ovules developed into seed when *V. elliottii* was the female parent and none of them germinated. When *V. corymbosum* was the female parent, no ovules developed if they were excised before 35 DAP. Only ovules placed in culture at late stages of embryo development resulted in complete seed development in culture and in subsequent seed germination.

TABLE 5. *In ovulo* culture of embryos from interspecific crosses in *Vaccinium* as affected by the stage of embryo development at the time of excision.

Days after pollination	Ovules placed in culture	Ovules that developed into seed		Seed germination	
V. elliottii (female) x *V. corymbosum* (male)	No.	No.	%	No.	%
15	225	3	1.3	0	0.0
22	150	2	1.3	0	0.0
29	150	3	2.0	0	0.0
V. corymbosum (female) x *V. elliottii* (male)					
14	250	1	0.4	0	0.0
21	250	0	0.0	-	-
28	375	0	0.0	-	-
35	375	0	0.0	-	-
42	375	10	2.7	2	20.0
49	375	3	0.8	3	100.0

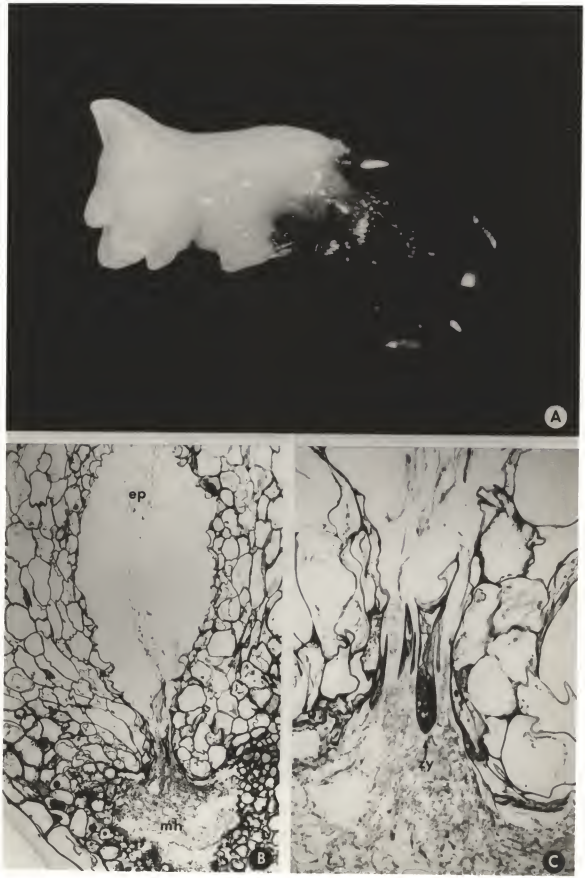
After ovules from interspecific crosses were placed in culture, little cell division occurred and growth was mainly due to a rapid increase in cell size, particularly at the internal layers of the integument. Ovules usually showed rapid cell enlargement during their first 2 weeks in culture, but did not show any other sign of growth or development from then on, even after they were transplanted into fresh medium. Since the epidermal cells of the ovule did not grow or divide, enlargement of the internal cells of the integument resulted in the rupture and cracking of the epidermis. In some cases, callus was formed from the internal cell layers of the integument, but in only one case was differentiation of this callus observed (Figure 11A). Placental tissues also showed some growth, and often callus was formed,

FIGURE 11. Cultured ovaries of *V. corymbosum* pollinated with *V. elliotti*.

A. Callus formed probably from the internal cell layers of the integument showing incipient differentiation. 15x.

B. Ovule sectioned after being 30 days in culture. Ovule was planted attached to the palcenta 17 days after pollination. Note the already disintegrated endosperm both in the area of the endosperm proper (ep) and in the area of the micropylar haustorium (mh). 87.5x.

C. Magnification of the same section showing the undivided zygote. 350x.


A
ep
mh
B
zy
C

particularly at the point where the placenta was attached to the ovary. Placental tissues remained green during most of the culturing period, and the callus that occasionally formed did not show any sign of differentiation.

Not all the ovules from a locule showed a similar response when placed in culture. Some of the ovules showed rapid growth after plating, while the majority did not show any sign of development and remained almost the same size they were are the time of plating. Probably only those ovules that were fertilized had the ability to develop. A similar situation was observed with ovules that developed *in vivo*.

Anatomical observations of cultured embryos from intraspecific crosses showed that the developmental pattern *in vitro* was similar to that *in vivo*. However, when ovules from interspecific crosses were cultured, the cells of the integument did not divide following endosperm degeneration, so that the remnants of the endosperm cells were not crushed at the center of the ovule (Figure 11B) as was the case when abortion occured *in vivo* (Figure 6I, 7E). Also, the zygote did not abort immediately following endosperm degeneration and instead remained alive but undivided. Figure 11C shows an example where hybrid ovules of *V. corymbosum*, placed in culture 17 DAP, still possess an undivided zygote even after being in culture for more than 30 days. Non-hybrid embryos were fully developed 47 DAP.

Treatments to delay embryo abortion before culturing the embryos have been used in other species to obtain interspecific hybrids (GOSAL and BAJAJ, 1983). Darrow (1956) first used this approach in blueberry with some success. In the experiments reported here,

application to the ovaries of 1% naphthalene acetamide in lanolin paste immediately after interspecific pollinations resulted in partial parthenocarpic fruit development, but there was no seed development. Naphthalene acetamide delayed the time of fruit abscission and induced the development of ovary walls at the sites where the paste was applied, but had no effect on ovule development. More than 2000 ovules of *V. corymbosum* were placed in culture 25 DAP following auxin treatment, but none developed into normal seed.

Addition of various media components, including coconut milk, sucrose, vitamins, and various growth regulators, resulted in a variety of different responses, none of which included the development of hybrid embryos. For example, an increase in sucrose concentration in the nutrient medium up to 50 g/l resulted in larger cells in the ovule, with no apparent increase in cell division. Concentrations above this level produced no further increase in cell size and concentrations of 120 g/l resulted in no growth at all. Vitamins added up to 3 times their normal concentration had no effect on the rate of ovule development in culture. Addition of coconut milk at the concentration used was also ineffective.

When growth regulators were added in various combinations to the nutrient medium, different responses were observed, but none of them increased the amount of hybrid embryos obtained. Addition of 2iP resulted in the production of large masses of callus from placental tissues. Ovules showed little growth and they were almost entirely covered by callus formed in all areas of the placenta. After 90 days in culture, callus completely covered the undeveloped ovules. Increasing 2iP concentrations resulted in the growth of a more organized

callus, particularly when 2,4-D was also present in the medium. Addition of 2,4-D was not essential for callus development, but when used at 0.5 mg/l there was a more rapid and abundant callus growth. Addition to the medium of Alar, a compound that has a reported antigibberellin action (KOCHBA et al.,1978), resulted in an increase in the final size attained by the ovules and callus growth from placental tissues was inhibited, particularly when 2iP was also added. Addition of ABA to the nutrient medium resulted in a very distinct effect. It completely suppressed the formation of placental callus and induced a rapid growth of the internal cells of the integument in such a way that the epidermis of the ovules was broken shortly after the ovules were plated.

Callus derived from ovules or from placental tissues did not show any sign of differentiation even after transfer to an auxin-free medium, except that a few roots were formed occasionally. This root differentiation was not in response to a particular treatment, but rather occurred at random among the different treatments.

In some species, it has been reported that when unfertilized ovules are placed in culture any cell of the embryo sac can form callus or directly give rise to an ovular haploid (YANG and ZHOU, 1982). In only one case was differentiation of callus from the ovule observed in the experiments reported here (Figure 11A), and even then the origin of the callus was probably not the embryo sac. Attempts to differentiate a whole plant from this callus were unsuccessful.

These data provide evidence that *in ovulo* embryo culture is feasible in *Vaccinium*, even at very early stages of embryo development. The failure to obtain viable hybrid embryos may have resulted from the

inability of the nutrient medium to provide a required developmental factor which was missing in the hybrid zygote. If hybrid zygotes started dividing and developed beyond the cotyledonary stage of embryo development, as occured when *V. corymbosum* was the female parent, ovules could successfully develop into normal seed in culture. This is an indication that there is an intrinsic developmental failure in some hybrid zygotes that can not be overcome by culturing the embryos in a simple culture medium.

In ovary Embryo Culture

Position of the ovary in the culture medium had a great effect on fruit development in culture. Experiments with intraspecific crosses in *V. corymbosum* demonstrated that when whole, undisturbed young fruits were placed in culture (Figure 10A) they showed little growth and differentiation (Table 6), although they remained green and alive for up to 40-50 days. On the contrary, fruit that were cut longitudinally (Figure 10C) before plating or were plated with the basal portion of the ovary removed (Figure 10B) usually developed into normal looking fruit.

This striking difference in behavior might have resulted from an inability of the components of the nutrient medium to diffuse into the internal tissues of the fruit unless the fruit was cut. The epidermis of both fruit and leaves of blueberries possesses heavy wax depositions (ALBRIGO et al.,1980), which are probably not removed during surface sterilization and therefore may constitute an important barrier to the diffusion of nutrients into the internal cells of the fruit. Cutting of the fruit puts a large number of mesocarp cells in direct

contact with the nutrient medium, and also brings large portions of the vascular system (BELL and GIFFIN, 1957) in closer contact with it, increasing the chances for rapid nutrient translocation to other parts of the fruit. An alternative explanation may be related to the wounding response induced by cutting the fruit. It is well established that tissue wounding induces various chemical changes in which several growth regulators are actively involved (KAHL, 1978).

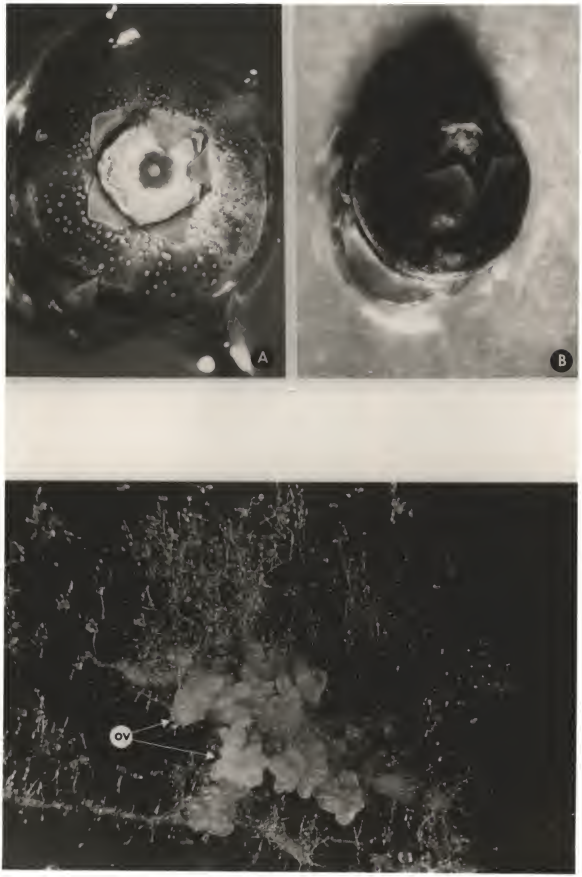
There was also an effect of the time at which ovaries were placed in culture. When fruit were cultured before the zygote started to divide, i.e. 10 DAP, they developed very few seed. When fruit were placed in culture 15 DAP, at a time when the embryos were past the globular stage of development, the number of viable seed that developed in culture was similar to the number of viable seed normally produced *in vivo* (ECK, 1966).

An almost identical response in fruit and seed development in culture was observed when ovaries of *V. elliottii* were placed in culture (data not presented). Based on these observations, all experiments with interspecific crosses were made using ovaries from which the basal third was removed before plating.

Fruit from intraspecific crosses developed in culture in a way similar to that in nature. Even the normal changes associated with fruit ripening were observed after the fruits completed their development in culture, which occurred about 40 days after plating (Figure 12A,B). Ripening was evidenced by a gradual change in the color of the exocarp from green to yellow and finally to dark blue. This change in color was accompanied by softening of the mesocarp, beakdown of the endocarp, and browning of the seed's testa. The

FIGURE 12. Fruit of *V. corymbosum* (A) and *V. elliottii* (B) that developed and ripened in culture.

FIGURE 13. *In vitro* pollinations. Ovules (ov) attached to the placenta were placed in a pollen germination medium were pollen germinated but failed to effect fertilization.


A
B
ov

frequency of successful fruit ripening in culture was unrelated to the time at which the ovaries were placed in culture and to the presence or absence of developing seed (Table 6).

Genetic differences in fruit developemnt period, i.e. days from full bloom to fruit ripening, did not express in culture in the same way they do *in vivo*. *V. elliottii* requires less days from full bloom to fruit ripening than *V. corymbosum* under normal growing conditions, however in culture, they started to ripen at about the same time.

TABLE 6. Fruit development in culture after intraspecific pollinations in *Vaccinium corymbosum*.

Days after pollination	Increase in fruit diameter[z] (mm)			Mature seed per fruit (No.)			Fruit ripening in culture		
	whole[y]	cross[x]	long.[w]	whole	cross	long.	whole	cross	long.
10	0.9	5.6	5.8	0.0	1.9	2.3	no	no	yes
15	0.8	5.4	5.2	3.2	19.5	11.5	no	yes	yes

[z]increase in fruit diameter from the time of plating to fruit maturity.

[y]whole: the whole fruit was plated.

[x]cross: the basal third of the fruit was cut before palting.

[w]long.: the fruit was longitudinally cut in halves before plating.

Tables 7 and 8 show the effect on seed production of the time at which the ovaries were placed in culture following interspecific pollinations. When *V. corymbosum* was the female parent (Table 7), 8.4%

of the plated ovaries developed an average of 1.7 seed, most of which were produced when ovaries were placed in culture between 14 and 18 DAP. Non-hybrid embryos were past the globular stage of embryo development 14 DAP.

TABLE 7. Seed development after crossing various clones and selections of *Vaccinium corymbosum* (female) and *V. elliottii* (male) as affected by the time at which ovaries were placed in culture.

Clones or selections crossed	Days after pollination	Ovaries plated	Ovaries with seed		Seed/ ovary
V.corymbosum x *V.elliottii*		No.	No.	%	No.
FL 5-12 x FL 82-226	11	18	1	5.6	1.0
FL 81-166 x FL 82-226	13	22	0	0.0	-
FL 79-25 x FL 82-226	13	20	0	0.0	-
Sharpblue x FL 82-226	14	26	5	19.2	2.0
NC-1688 x FL 82-226	15	26	0	0.0	-
Avonblue x FL 82-226	18	36	6	16.7	1.7
FL 64-76 x FL 82-226	18	5	1	20.0	1.0
Flordablue x FL 82-226	18	20	3	15.0	1.7
FL 79-26 x FL 82-226	19	12	0	0.0	-
FL 64-76 x FL 82-226	24	6	0	0.0	-
Total	11-24	191	16	8.4	1.7

When *V. elliottii* was the female parent (Table 8), 5.0% of the plated ovaries developed seed, averaging 1 seed per fruit. The time at which the ovaries were placed in culture seemed to be less critical than when *V. corymbosum* was the female parent.

The effect of the genotype of the female parent on hybrid seed production was difficult to establish. When *V. elliottii* was used as male parent, there was large variability in pollen viability and in the effectiveness of the pollination process (see Results, Section III),

TABLE 8. Seed development after crossing various clones and selections of *Vaccinium elliottii* (female) and *V. corymbosum* (male) as affected by the time at which ovaries were placed in culture.

Clones or selections crossed	Days after pollination	Ovaries plated	Ovaries with seed		Seed/ ovary
V.elliottii x *V.corymbosum*		No.	No.	%	No.
FL 81-198 x FL 82-229[z]	12	24	1	4.2	1.0
FL 81-198 x FL 82-229	14	48	2	4.2	1.0
FL 82-178 x FL 82-229	14	22	3	13.6	1.0
FL 82-145 x FL 82-229	15	37	3	13.6	1.0
FL 82-147 x FL 82-229	17	39	0	0.0	-
FL 82-178 x FL 82-229	19	26	1	3.8	1.0
FL 80-69 x FL 82-229	21	36	2	5.6	1.0
FL 80-69 x FL 82-229	24	10	0	0.0	-
Total	12-24	242	12	5.0	1.0

[z]FL 82-229 is a pollen mixture of 'Sharpeblue', 'Avonblue', 'Flordablue', NC 1688, and FL 64-76.

therefore the effect of the female genotype could not be independently estimated.

It has already been mentioned that in some species addition of particular compounds to the nutrient medium can greatly increase the rate at which embryos can be obtained in culture (NORSTOG and SMITH, 1963; PHILLIPS, 1981; RAGHAVAN and SRIVASTAVA, 1982). Since none of the compounds tested in the *in ovulo* experiments enhanced the ability of the hybrid embryos to grow in culture, a different group of chemicals was tested for the *in ovary* experiments. Norstog (1979), in his review of embryo culture techniques, cited a number of cases where particular amino acids have been found essential for the successful culture of some embryos. Although casein hydrolysate, one of the components of the

nutrient medium used, contains all the essential amino acids, several experiments were conducted incorporating additional amounts of some of the amino acids that have been claimed to be essential. Addition of alanine, glutamine, proline, or a combination of all 3 did not increase the number of hybrid seed obtained, nor did they have an effect on the developmental pattern of the cultured ovaries.

Various sucrose concentrations were also tested. Although sucrose did not have any effect on the development of hybrid seed, there was a marked effect on fruit ripening in culture. When the sucrose concentration in the nutrient medium was below 30 g/l, the fruit did not change color even after 90 days in culture. Fruit growth, however, was comparable to that obtained when the sucrose concentration was 30 g/l or higher. This observation suggests that anthocyanin accumulation, known to be responsible for color development in blueberry (SAPERS et al.,1984), occurred only when a certain minimum level of sucrose was provided. Sugar accumulation in the fruit has been proposed as the controlling factor in the initiation of berry ripening in grape (COOMBE, 1960). Other changes associated with ripening, such as mesocarp softening, occurred later in low-sucrose medium than in a medium containing 30 g/l or more.

As in the case of *in ovulo* embryo culture, addition of growth regulators to the nutrient medium did not augment the ability of hybrid seed to develop in culture. A tendency to differentiate callus from ovary walls and from the sepals was observed when either 2iP and/or picloram were present in the nutrient medium. Little callus formation was observed when no growth regulators were added to the nutrient medium.

Again, evidence presented here suggests that, since non-hybrid embryos can be cultured at proembryonic stages of development without removing them from the ovary, an intrinsic developmental failure causes abortion of hybrid seed.

We have not yet done cytological studies to assess the chromosomal constitution of the plants obtained either through *in ovulo* or *in ovary* embryo culture. Lyrene and Sherman (1983) have determined that *V. corymbosum* x *V. elliottii* hybrids obtained *in vivo* included triploids, tetraploids, pentaploids, and aneuploids and that some hybrids appeared to be mosaics of various chromosome numbers.

In Vitro Pollinations

It has been already mentioned that, when *V. elliottii* was the male parent, pollen grains germinated and grew down the style but failed to grow into the locules of the ovary and effect fertilization (see Results, Section III). *In vitro* pollination techniques have been used in a number of species to overcome prefertilization barriers to hybridization (ZENKTELER, 1980). A few experiments were conducted using these techniques in an effort to increase the number of pollen tubes that can effect fertilization, particularly when *V. elliottii* was used as male parent. *In vitro* pollination, as used in other species, consists of growing naked ovules in a nutrient medium suitable for pollen germination, onto which pollen grains are directly placed (ZENKTELER, 1980).

Application of this technique using both intra- and interspecific combinations of parents, resulted in pollen germination

and pollen tube growth, but pollen tubes failed to find the micropyle and penetrate the ovules (Figure 13). Using a liquid medium and floating the ovules on its surface did not improve the situation. Profuse pollen tube growth was obtained, but pollen tubes failed to find the micropyle of the ovules.

It has been proposed that an orientation mechanism exists that directs pollen tube growth through the style and to the micropyle, but evidence for such a mechanism is only circumstantial for most species (KAPIL and BHATNAGAR, 1975). A chemotropic substance is presumably produced by the synergid and secreted through the filiform apparatus (BERGER AND ERDELSKA, 1973). Different compounds have been proposed as chemotropic, but a calcium gradient seems to be the most frequently cited factor in directing pollen tube growth (KAPIL and BHATNAGAR, 1975). Since it was obvious that the orientation mechanism was not working under the experimental conditions used for *in vitro* pollination, additional experiments were conducted in which whole flowers were placed in culture immediately following emasculation and artificial pollination. Observation of the pistils of these flowers 48 h after pollination demonstrated that pollen had germinated and that pollen tubes had grown to the base of the style. When pollen tube growth was checked 48 h later, it was observed that the tubes had not grown any further and had failed to penetrate the ovarian cavity since they were not observed growing on the surface of the placenta as in normal pollinations.

Since pollen tubes were arrested before penetrating the ovarian cavity in both intra- and interspecific pollinations, it was clear that the orientation mechanism was not operative under culture

conditions and that tissues other than those of the ovules may be involved in directing pollen tubes to the micropyle. It is well documented that in some species fruit set is strongly influenced by environmental factors (LEOPOLD and KRIEDEMAN, 1975). Environmental conditions for fruit set in blueberry are not well defined, however, it has been suggested that fruit set is affected by temperatures prevailing following pollination (MEADER and DARROW, 1947). Requirements for *in vitro* fruit set must be established before *in vitro* pollinations can be used in blueberry.

SECTION V

SUMMARY AND CONCLUSIONS

1. A strong interspecific cross-incompatibility exists that prevents the formation of hybrid plants in crosses between *Vaccinium elliottii*, a diploid species, and *V. corymbosum*, a tetraploid species. This incompatibility was observed regardless of the direction of the cross.

2. Pre- and postfertilization barriers were detected. Prefertilization barriers manifested as an arrest in pollen tube growth at the base of the style, so that pollen tubes failed to penetrate the ovarian cavity to effect fertilization. Prefertilization barriers were observed only when *V. elliottii* was the male parent.

3. In those cases where fertilization occurred normally, in either *V. corymbosum* x *V. elliottii* or its reciprocal cross, a strong postfertilization barrier induced embryo abortion soon after gamete fusion. The zygote remained in a resting stage following fertilization and usually aborted before dividing. The endosperm

went into 4-6 cycles of cell division before it started to disintegrate. Endosperm malfunction and embryo abortion were delayed when *V. corymbosum* was the female parent. A causal relationship between endosperm and embryo abortion was not established.

4. Attempts to overcome the incompatibility using *in ovulo* and *in ovary* embryo rescue resulted in the production of a few presumably hybrid seeds when a rather simple culture medium was used. Addition of various vitamins, amino acids, and growth regulators to the medium did not increase the number of seed produced. An intrinsic developmental failure seemed to prevent the division of hybrid zygotes, since non-hybrid seed was obtained even when embryos were placed in culture soon after fertilization. Furthermore, abortion was delayed and the zygote remained alive but undivided in cultured embryo sacs.

5. After intraspecific pollinations, viable non-hybrid seed was obtained with both *in ovary* and *in ovulo* embryo culture techniques when embryos were placed in culture at proembryonic stages of development. A simple culture medium containing no growth regulators was used. Successful seed development in culture was obtained only when ovules were cultured attached to placental tissues. When *in ovary* embryo culture was used, fruit and seed development in culture occurred only

when the fruit was cut either longitudinaly or when the basal portion of it was removed previous to plating, and the cut surface was placed in direct contact with the nutrient medium.

6. Attempts to increase the number of fertilized eggs using *in vitro* pollination techniques in both intra- and interspecific pollinations failed because, in spite of good pollen germination and tube growth, pollen tubes failed to penetrate the ovarian cavity.

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BIOGRAPHICAL SKETCH

Carlos E. Muñoz Schick was born January 6, 1948, in Santiago, Chile. He received the degree of Ingeniero Agronomo from the University of Chile in March, 1971. After graduation he worked for 3 years as associate researcher for a development program at the Atacama desert in northern Chile. Later he worked as a fruit crops extension agent for a growers' cooperative and as production manager for a mushroom growing farm in the central part of the country.

From 1977 to 1979 he was a researcher in the Fruit Crops Department at the Instituto de Investigaciones Agropecuarias in Santiago. In 1979 he was awarded a scholarship from the United Nations Development Program and the InterAmerican Development Bank to study in the United States of America.

He enrolled as a graduate student in the Fruit Crops Department at the University of Florida in January, 1980. He received the degree of Master of Science in December, 1981, working in the area of plant physiology.

In January, 1982, he enrolled again in the Fruit Crops Department at the same University as a Ph. D. student, this time in the area of fruit breeding.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul M Lyrene

Paul M. Lyrene, Chairman
Associate Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Wayne B. Sherman

Wayne B. Sherman
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Mark J. Bassett

Mark J. Bassett
Associate Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gloria A. Moore

Gloria A. Moore
Assistant Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Indra K. Vasil
Graduate Research Professor of Botany

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1984

Dean, College of Agriculture

Dean for Graduate Studies and Research